

National Institute of Child Health and Human Development

NICHD

ANNUAL REPORT

FY95

October 1, 1994 to September 30, 1995

PART 1

J

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V277

1995

pt. 1

**PROJECT NUMBER LISTING
for the
ANNUAL REPORT
of the
NICHD**

October 1, 1994 - September 30, 1995

Project Numbers - Annual Report 1995

BIOMETRY BRANCH (BB)

ZO1 HD 00841-13 TERMINATED	Methods for Comparing and Analyzing Data from Several Complex Surveys Kai F. Yu, Ph.D.
ZO1 HD 00855-04	Statistical Methods for the Common Odds Ratio of a Number of Contingency Tables Kai F. Yu, Ph.D.
ZO1 HD 00856-04	Statistical Methods for a Mixture of Subpopulations Kai F. Yu, Ph.D.
ZO1 HD 00857-04	Methodological Research in Mathematical Statistics and Biostatistics Young J. Lee, Ph.D.
ZO1 HD 00859-04	Meta-Analytic Methods Rebecca DerSimonian, Ph.D.

Z01 HD 00864, 00866-869

Project Numbers - Annual Report 1995

CELL BIOLOGY AND METABOLISM BRANCH (CBMB)

Z01 HD 01600-11	Biochemical Basis of T Cell Activation Larry E. Samelson, M.D.
Z01 HD 01602-11	Regulation of Intracellular Iron Metabolism Tracey A. Rouault, M.D.
Z01 HD 01606-07	The Biology of Early Organelles of the Secretory Pathway Richard D. Klausner, M.D.
Z01 HD 01607-05	Protein Trafficking in the Secretory Pathway Juan Bonifacino, Ph.D.
Z01 HD 01608-05	Gene Regulation in Response to Environmental Stress Gisela Storz, Ph.D.
Z01 HD 01609-04	Localization and Dynamics of Intracellular Organelles Jennifer Lippincott-Schwartz, Ph.D.
Z01 HD 01610-03	Intracellular Metal Metabolism Richard D. Klausner, M.D.
Z01 HD 01611-01	The VHL Tumor Suppressor Gene Richard D. Klausner, M.D.

Z01 HD 01612-99

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COMPUTER SCIENCES BRANCH (CSB)

ZO1 HD 01802-05

Data Coordinating Center for the NICHD Study of Early Child Care
Bonnie K. Knoke

Project Numbers - Annual Report 1995

DEVELOPMENTAL ENDOCRINOLOGY BRANCH (DEB)

ZO1 HD 00610-15	Growth, Puberty, Their Disorders: Physiology, Pathophysiology, and Molecular Biology Gordon B. Cutler, Jr., M.D.
ZO1 HD 00615-15	Endocrine-Immune Interactions George P. Chrousos, M.D.
ZO1 HD 00616-14 TERMINATED	Structure, Function, and Physiology of Glycoprotein Hormones Bruce C. Nisula, M.D.
ZO1 HD 00618-14	Physiology and Pathophysiology of the Hypothalamic-Pituitary-Adrenal Axis George P. Chrousos, M.D.
ZO1 HD 00623-12	Adrenal Physiology, Pathophysiology, and Molecular Biology Gordon B. Cutler, Jr., M.D.
ZO1 HD 00627-06	Glycoprotein Hormones: Oligosaccharide Structure and Function Diana Blithe, Ph.D.
ZO1 HD 00628-06	Biological Roles and Mechanisms of Action of Insulin-Like Growth Factors (IGFs) Carolyn Bondy, M.D.
ZO1 HD 00631-06	Neuroendocrine Control of the Stress Reponse Greti Aguilera, M.D.
ZO1 HD 00632-06	Physiological Actions of the Renin-Angiotension-System Greti Aguilera, M.D.
ZO1 HD 00633-05	Ovarian Folliculogenesis Lawrence Nelson, M.D.
ZO1 HD 00634-03 TERMINATED	Metabolic Effects of Insulin-like Growth Factor I in Normal and Diabetic Adolescents Carolyn Bondy, M.D.
ZO1 HD 00636-03	Endocrinology of Reproduction in Women James Segars, M.D.
ZO1 HD 00637-02	Steroid Hormone Action in Female Reproduction Lynnette Nieman, M.D.
ZO1 HD 00638-02	Physiology of Hypercortisolism Lynnette Nieman, M.D.

ZO1 HD 00639-99

DIVISION OF EPIDEMIOLOGY, STATISTICS AND PREVENTION RESEARCH (DESPR)

- Z01 HD 01703-06 Study of Pregnancy Outcome, Maternal Death and Child Health in Pakistan
Heinz W. Berendes, M.D., M.H.S.
- Z01 HD 01704-05 Stunting Among Bedouin Arab Children in the Negev, Israel
PRJT. COMPLETED (10/1-31/94) Heinz W. Berendes, M.D., M.H.S.
- Z01 HD 01705-02 Women's Lifestyles in Pregnancy Study, Analysis of Data
PRJT. COMPLETED (10/1-12/1/94) Heinz W. Berendes, M.D., M.H.S.
- Z01 HD 01706-01 A Randomized Controlled Trial for the Evaluation of a New Antenatal Care Model
Heinz W. Berendes, M.D., M.H.S.

Z01 HD 01707-99

ENDOCRINOLOGY AND REPRODUCTION RESEARCH BRANCH (ERRB)

ZO1 HD 00035-23	The Structure and Function of Peptides and Proteins Hao-Chia Chen, Ph.D.
ZO1 HD 00146-19 TERMINATED	Structural Studies of Proteins Hao-Chia Chen, Ph.D.
ZO1 HD 00147-19 TERMINATED	Mechanism of Action of Peptide Hormones in Steroidogenic Cells Maria L. Dufau, M.D., Ph.D.
ZO1 HD 00150-20	Characterization of Gonadal Receptors and Mechanisms of Action of Peptide Hormones in Steroidogenic Cells Maria L. Dufau, M.D., Ph.D.
ZO1 HD 00184-17	Regulation of Pituitary Hormone Secretion Kevin J. Catt, M.D., Ph.D.
ZO1 HD 00187-16	Hormonal Regulation of Cellular Metabolism Kuo-Ping Huang, Ph.D.
ZO1 HD 00193-10	Angiotensin II Receptors and Activation Mechanisms Kevin J. Catt, M.D., Ph.D.
ZO1 HD 00194-07	Steroid Biosynthesis and Metabolism in the Mammalian Adrenal Cortex Charles A. Strott, M.D.
ZO1 HD 00195-02	Intracellular Signalling in Endocrine Cells Stanko Stojilkovic, Ph.D.

ZO1 HD 00196-301

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EPIDEMIOLOGY BRANCH (EB)

ZO1 HD 00331-12	Diabetes in Early Pregnancy (DIEP) James L. Mills, M.D., M.S.
ZO1 HD 00334-12	Low Birth Weight Across Generations Mark A. Klebanoff, M.D., M.P.H.
ZO1 HD 00361-09	Child Health Supplement to the 1988 National Health Interview Survey Mary D. Overpeck, M.P.H.
ZO1 HD 00369-07	Adverse Perinatal Events and Subsequent Injury-related Death Mark A. Klebanoff, M.D., M.P.H.
ZO1 HD 00373-07	Calcium Supplementation in Pregnancy for the Prevention of Preeclampsia Richard J. Levine, M.D.
ZO1 HD 00379-06	Data Analysis from the Vaginal Infections and Prematurity Study Mark A. Klebanoff, M.D., M.P.H.
ZO1 HD 00382-05 PRJT. COMPLETED (10/1/94-2/28/95)	Cocaine and Marijuana Use During Pregnancy and Pregnancy Outcome Mark A. Klebanoff, M.D., M.P.H.
ZO1 HD 00383-05	Analyses of Data from the Collaborative Perinatal Project Mark A. Klebanoff, M.D., M.P.H.
ZO1 HD 00384-04	Field Trial of Oral Cholera Vaccines in Bangladesh John D. Clemens, M.D.
ZO1 HD 00385-04	Epidemiology of Rotavirus Infections in Bangladesh John D. Clemens, M.D.
ZO1 HD 00386-04	Evaluation of a Water-Sanitation Intervention in Egypt John D. Clemens, M.D.
ZO1 HD 00389-04	Studies of the Epidemiology of Pediatric Shigellosis in Bangladesh John D. Clemens, M.D.
ZO1 HD 00392-04	Fatal Injuries to U.S. Infants, 1983-87 Ruth A. Brenner, M.D., M.P.H.
ZO1 HD 00393-04 PROJ. COMPLETED (10/1/94-5/31/95)	Trends in Death Rates from Drowning Among Children, 1971-88 Ruth Brenner, M.D., M.P.H.
ZO1 HD 00801-20	Studies Based on the Medical Birth Registries of Norway and Sweden Allen A. Herman, M.D., Ph.D.
ZO1 HD 00861-13	Assessment of In-Utero Fetal Growth Patterns in Relation to Outcome at Birth Allen A. Herman, M.D., Ph.D.

Continued →

EPIDEMIOLOGY BRANCH (EB)

(continued)

Z01 HD 00872-10	Factors Associated with Premature Births: Missouri Follow-Back Survey Allen A. Herman, M.D., Ph.D.
Z01 HD 2500-03	ETEC Seroepidemiology Evaluations in Alexandria, Egypt John D. Clemens, M.D.
Z01 HD 2502-03	NICHD-Health Research Board of Ireland Neural Tube Defects Study James L. Mills, M.D., M.S.
Z01 HD 2503-03	1991 Follow-up National Maternal and Infant Health Survey PROJ. COMPLETED (10/1/94-3/31/95) Mary D. Overpeck, M.P.H.
Z01 HD 2505-03	Infectious Disease Mortality During Infancy: United States, 1987 PROJ. COMPLETED (10/1-12/31/94) Jennifer S. Read, M.D., M.P.H.
Z01 HD 2506-03	Lack of Age-Appropriate Immunizations Among Infants Born in D.C. Ruth A. Brenner, M.D., M.P.H.
Z01 HD 2507-03	Prostaglandin Excretion in Preeclampsia James L. Mills, M.D., M.S.
Z01 HD 2510-03	Diet, Maternal Nutritional Status, Blood Pressure and Fetal Growth Nebiat Tafari, M.D.
Z01 HD 2511-02	Immunogenicity of Routine Childhood Vaccines in HIV Positive Children Jennifer S. Read, M.D.
Z01 HD 2512-02	Birth Certificate Linkage to Growth and Health Measures Using NHANES III Mary D. Overpeck, M.P.H.
Z01 HD 2513-02	High Status and Risk Factors Analysis of Premature High Risk Deliveries Mary D. Overpeck, M.P.H.
Z01 HD 2514-02	Childhood Drowning Deaths - A National Analysis of Death Certificate Information Ruth A. Brenner, M.D., M.P.H.
Z01 HD 2515-02	Prevention of Childhood Injuries - Phase I Injury Surveillance Ruth A. Brenner, M.D., M.P.H.
Z01 HD 2516-02	The Role of Bathtub Rings and Seats in Infant Drowning Deaths Ruth A. Brenner, M.D., M.P.H.
Z01 HD 2517-02	First Week Changes in Birthweight and Other Neonatal Anthropometric Parameters Nebiat Tafari, M.D.

Continued →

EPIDEMIOLOGY BRANCH (EB)

(continued)

Z01 HD 2518-02	Effectiveness of Rapid Plasma Reagin Screening in Gestational Syphilis Nebiat Tafari, M.D.
Z01 HD 2519-01	Determination of Protection Level of Maternal Antibody to Group B Streptococcus Feng-Ying Lin, M.D., M.P.H.
Z01 HD 2520-01	Maternal Caffeine Use in Pregnancy Outcome Mark A. Klebanoff, M.D., M.P.H.
Z01 HD 2521-01	Replacement of Pregnancy Losses: Interpregnancy Interval in Adolescence Yvette R. Johnson, M.D., M.P.H.
Z01 HD 2522-01	Intrapartum Antibiotic Prophylaxis Against Early-Onset Group B Streptococcal Disease Yvette R. Johnson, M.D., M.P.H.
Z01 HD 2523-01	Studies Based on Maternally Linked Birth Registries of Missouri and Utah Allen A. Herman, M.D., Ph.D.
Z01 HD 2524-01	Occupational Nonfatal Injuries in U.S. Children Mary D. Overpeck, M.P.H.
Z01 HD 2525-01	World Health Organization Study of Health Behavior in School Children (WHO-HBSC) Mary D. Overpeck, M.P.H.

Z01 HD 002526-2600

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HERITABLE DISORDERS BRANCH (HDB)

ZO1 HD 00131-21	Human Biochemical Genetics William A. Gahl, M.D., Ph.D.
ZO1 HD 00137-21	Regulation and Expression of the UDP-Glucuronosyltransferase Gene Family Ida S. Owens, Ph.D.
ZO1 HD 00404-13	Cell and Sulfur Metabolism in Fibroblasts of Genetic Diseases Jean DeBrohun Butler, Ph.D.
ZO1 HD 00408-12	Heritable Disorders of Connective Tissue Joan C. Marini, M.D., Ph.D.
ZO1 HD 00415-04 (Merged with Z01 HD 00910-16)	Physiology and Molecular Biology of Arachidonate Metabolism and CFTR Mutation in CF Anil B. Mukherjee, M.D., Ph.D.
ZO1 HD 00417-04 TERMINATED	Molecular Basis of alpha 1-Antitrypsin Deficiency Mark Brantly, M.D.
ZO1 HD 00910-16	Study of Uteroglobin, Phospholipase A ₂ , and Osteopontin Genes Anil B. Mukherjee, M.D., Ph.D.
ZO1 HD 00912-16	Gene Regulation and Cellular Differentiation Janice Y. Chou, Ph.D.

ZO1 HD 00139-45
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Project Numbers - Annual Report 1995

LABORATORY OF COMPARATIVE ETHOLOGY (LCE)

ZO1 HD 01106-12	Developmental Continuity of Individual Differences in Reactivity in Monkeys Stephen J. Suomi, Ph.D.
ZO1 HD 01107-12	Adaptation of Laboratory Reared Monkeys to Field Environments Stephen J. Suomi, Ph.D.
ZO1 HD 01112-09	Effects of Home and Out-of-Home Care on Child Development Michael E. Lamb, Ph.D.
ZO1 HD 01114-08	Individual Differences in Physical and Affective Functioning in Infancy Michael E. Lamb, Ph.D.
ZO1 HD 01115-08	Effects of Domestic Violence and Evaluation of Children's Testimony Michael E. Lamb, Ph.D.
ZO1 HD 01116-08	Social Development Across the Lifespan in Diverse Cultures and Ecologies Michael E. Lamb, Ph.D.
ZO1 HD 01117-08	The Hospitalization Experience: Children's Coping with the Stress of Surgery Marc H. Bornstein, Ph.D.
ZO1 HD 01119-08	Specificity of Mother-Infant Interaction Marc H. Bornstein, Ph.D.
ZO1 HD 01120-08	Parenting and Infancy Activity: A Multiculture Perspective Marc H. Bornstein, Ph.D.
ZO1 HD 01122-08	Longitudinal Assessment of Children's Mental and Social Abilities Marc H. Bornstein, Ph.D.
ZO1 HD 01123-05	Physiological Correlates and Neural Mechanisms of the Infant Cry and Related Vocalizations John Newman, Ph.D.
ZO1 HD 01124-05	Genetic and Experiential Influences on the Development of Primate Vocal Behavior John Newman, Ph.D.
ZO1 HD 01125-02 TERMINATED	Psychophysiological Substrates of Cognitive Processing and Socioemotional Expression from 2 to 36 Months Marc H. Bornstein, Ph.D.

ZO1 HD 01126-1199

LABORATORY OF CELLULAR AND MOLECULAR NEUROPHYSIOLOGY (LCMN)

- ZO1 HD 00707-11 Pharmacological Studies of Synaptic Transmission In Vitro
Mark L. Mayer, Ph.D.
- ZO1 HD 01205-03 Cellular and Synaptic Physiology of Hippocampal Interneurons
Chris J. McBain, Ph.D.
- ZO1 HD 01206-02 Receptor Mediated Calcium Signalling in Glia and Neurons
James T. Russell, D.V.M., Ph.D.
- ZO1 HD 02000-04 Neurotransmitter Receptors in Glia
Vittorio Gallo, Ph.D.

ZO1 HD 01207-99

LABORATORY OF DEVELOPMENTAL AND MOLECULAR IMMUNITY (LDMI)

ZO1 HD 01301-13	Human Immune Response to Polysaccharide-Protein Conjugate Vaccines Rachel Schneerson, M.D.
ZO1 HD 01308-12	Conjugate-Induced Polysaccharide Antibodies Shousun C. Szu, Ph.D.
ZO1 HD 01311-02	A Polysaccharide Vaccine for a Mycobacterium-Tuberculosis John B. Robbins, M.D.
ZO1 HD 01312-01	Isolation and Purification of Subunit B of Shiga Toxin Vince Pozsgay, Ph.D.
ZO1 HD 01313-01	Synthetic Vaccine Against Shigellosis Vince Pozsgay, Ph.D.
ZO1 HD 01314-01	Analysis and Synthesis of Carbohydrate Antigens of Mycobacterium Tuberculosis Vince Pozsgay, Ph.D.
ZO1 HD 01315-01	Modulation of Protein and Cell Functions by Heparin/Heparan Sulfates Audrey L. Stone, Ph.D.

ZO1 HD 01316-99

LABORATORY OF DEVELOPMENTAL NEUROBIOLOGY (LDN)

ZO1 HD 00047-26	Biochemical Studies of Neurons and Other Cell Types Douglas E. Brenneman, Ph.D.
ZO1 HD 00056-20	Biosynthesis, Processing and Secretion of Neuropeptides and Pituitary Peptide Hormones Yoke Peng Loh, Ph.D.
ZO1 HD 00064-19	Neurobiologic Studies of Neurons and Glia in Cell Culture Phillip G. Nelson, M.D., Ph.D.
ZO1 HD 00094-25	Pineal Regulation: Environmental and Physiology Factors David C. Klein, Ph.D.
ZO1 HD 00095-25	Pineal Regulation: Transsynaptic and Intracellular Mechanisms David C. Klein, Ph.D.
ZO1 HD 00704-11	Tetanus Toxin Effects and Localization in Neurons Elaine A. Neale, Ph.D.
ZO1 HD 00708-11	Morphological Studies of Neuronal and Non-Neuronal Cells in CNS Cell Cultures Elaine A. Neale, Ph.D.
ZO1 HD 00710-07	Molecular Characterization of Glutamate Receptor Expression in Brain Andres Buonanno, Ph.D.
ZO1 HD 00711-06	Transcriptional Regulation of Skeletal Muscle-Specific Genes by Electrical Activity Andres Buonanno, Ph.D.
ZO1 HD 00712-04	Regulation of Phenotypic Differentiation in the Developing Mammalian CNS Denes Agoston, M.D.
ZO1 HD 00713-01	Regulation of Gene Transcription and Neurite Outgrowth by Neural Impulse Activity Richard D. Fields, Ph.D.
ZO1 HD 01202-07 TERMINATED	Regulation of Expression and Function of Neuropeptides During Development Yoke Peng Loh, Ph.D.

ZO1 HD 00714-799

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LABORATORY OF EUCARYOTIC GENE REGULATION (LEGR)

Z01 HD 01004-12	Regulation of Amino Acid and Nucleotide Biosynthesis in <i>Saccharomyces Cerevisiae</i> Alan G. Hinnebusch, Ph.D.
Z01 HD 01009-03	Regulation and Function of Genetic Elements Henry Levin, Ph.D.
Z01 HD 01010-01	Regulation of Eukaryotic Protein Synthesis Thomas E. Dever, Ph.D.

Above project numbers are also included in the FY95 listing for the Laboratory of Molecular Genetics (LMG).

Project Numbers - Annual Report 1995

LABORATORY OF MOLECULAR EMBRYOLOGY (LME)

- | | |
|-----------------|--|
| Z01 HD 01900-05 | Developmental Regulation of Differential Gene Expression
Alan Wolffe, M.D. |
| Z01 HD 01901-01 | Gene Regulation by Thyroid Hormone during Tissue Remodeling
Yun-Bo Shi, Ph.D. |
| Z01 HD 01902-01 | Analysis of the S Phase Checkpoint in Higher Eukaryotes
Mary Dasso, Ph.D. |

Z01 HD 01903-99

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LABORATORY OF MOLECULAR GENETICS (LMG)

- ZO1 HD 00066-25 Control Mechanisms in Temperate Bacteriophage Lambda
Robert A. Weisberg, Ph.D.
- ZO1 HD 00067-27 The Integration of Macromolecular Synthesis in Escherichia coli
Michael Cashel, M.D., Ph.D.
- ZO1 HD 00068-24 Factors Influencing Genetics Transcription-Initiation and Termination
Robert J. Crouch, Ph.D.
- ZO1 HD 00069-23 Molecular Genetics of Mammalian Retrovirus Replication
Judith G. Levin, Ph.D.
- ZO1 HD 01002-13 Gene Expression During Embryonic Development of *Xenopus Laevis*
Igor B. Dawid, Ph.D.
- ZO1 HD 01004-12 Regulation of Amino Acid and Nucleotide Biosynthesis in *Saccharomyces Cerevisiae*
Alan G. Hinnebusch, Ph.D.
- ZO1 HD 01005-08 Regulation of Cellular Proliferation and Diversity in *Drosophila*
James A. Kennison, Ph.D.
- ZO1 HD 01006-07 Protein-Nucleic Acid Interactions in Vertebrate Embryogenesis
Thomas D. Sargent, Ph.D.
- ZO1 HD 01008-06 Molecular Genetics of Protein-Nucleic Acid Interactions in *Drosophila*
Susan R. Haynes, Ph.D.
- ZO1 HD 01009-03 Regulation and Function of Genetic Elements
Henry Levin, Ph.D.
- ZO1 HD 01010-01 Regulation of Eukaryotic Protein Synthesis
Thomas E. Dever, Ph.D.

ZO1 HD 01011-99

LABORATORY OF MAMMALIAN GENES AND DEVELOPMENT (LMGD)

Z01 HD 00071-23 Gene and Transgene Regulation in the Developing Mouse
Heiner Westphal, M.D.

Z01 HD 01800-05 The Genetic Basis of Mammalian Kidney Development
TERMINATED Gregory Dressler, Ph.D.

Z01 HD 01801-06 The Molecular Analysis of Murine Development
Kathy Mahon, Ph.D.

Z01 HD 01803-02 Genetic Analysis of Thymocyte Development
Paul Love, M.D.

Z01 HD 01804-99

LABORATORY OF MOLECULAR GROWTH REGULATIONS (LMGR)

Z01 HD 00412-08	Molecular Regulation of Gene Expression Richard J. Maraia, M.D.
Z01 HD 00505-02	Eukaryotic Transcriptional Regulation Yoshihiro Nakatani, Ph.D.
Z01 HD 01310-09	Developmental Gene Regulation of the Immune System Keiko Ozato, Ph.D.
Z01 HD 08719-15	Development and Uses of Eukaryotic Vectors Bruce H. Howard, M.D.

Z01 HD 00506-599

LABORATORY OF THEORETICAL AND PHYSICAL BIOLOGY (LTPB)

ZO1 HD 00165-20	Isolation and Characterization of Macromolecular and Cellular Particles Andreas C. Chrambach, Ph.D.
ZO1 HD 00171-19	Electrophoretic Methodology Andreas C. Chrambach, Ph.D.
ZO1 HD 01400-13	Dynamics of the Growth and Development of Bone Alfred L. Yergey, Ph.D.
ZO1 HD 01408-04 TERMINATED	Stability and Specificity of DNA-Protein Interactions Mark M. Garner, Ph.D.
ZO1 HD 01409-10	Membrane Transport and Fusion Joshua Zimmerberg, M.D., Ph.D.
ZO1 HD 01415-05	Kinetics of Exocytosis Joshua Zimmerberg, M.D., Ph.D.
ZO1 HD 01416-03	Tissue Imaging in Cell Biology Leonid Margolis, Ph.D.
ZO1 HD 01417-02	Energetics of the Interaction Between Water, Membranes, and Macromolecules Alfred L. Yergey, Ph.D.
ZO1 HD 01418-02	Regulation of Mitosis at the Cellular Level Frank A. Suprynowicz, Ph.D.
ZO1 HD 01419-02	Localization of Membrane Fusion Proteins Joshua Zimmerberg, M.D., Ph.D.
ZO1 HD 01420-01	Calcium Triggered Membrane Trafficking in Sea Urchin Eggs Steven Vogel, Ph.D.
ZO1 HD 01501-04	Role of Lipids in Membrane Rearrangements Leonid V. Chernomordik, Ph.D.

ZO1 HD 01421-99

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OFFICE OF THE SCIENTIFIC DIRECTOR (OSD)

ZO1 HD 00093-21	Mechanism of Action of Nerve Growth Factor Gordon Guroff, Ph.D.
ZO1 HD 01500-13	Studies on DNA Replication, Repair, and Mutagenesis in Eukaryotic and Prokaryotic Cells Arthur S. Levine, M.D.

ZO1 HD 01502-99

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PERINATOLOGY BRANCH (PB)

Z01 HD 02400-04 The Role of Subclinical Infection and Cytokines in Preterm Parturition
Roberto Romero, M.D.

Z01 HD 02401-03 Prenatal Diagnosis of Congenital Anomalies
Roberto Romero, M.D.

Z01 HD 2402-99

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PREVENTION RESEARCH BRANCH (PRB), DESPR

ZO1 HD 00876-05	Determinants of Childhood Poison Ingestion Lois A. Maiman, Ph.D.
ZO1 HD 00877-05	Prevention and Education in Coronary Heart Disease Reduction Lois A. Maiman, Ph.D.
ZO1 HD 2100-04	Prenatal Care Utilization and Adverse Health Behaviors During Pregnancy Lois A. Maiman, Ph.D.
ZO1 HD 2101-04	Increasing Preventive Actions for Childhood Injury Among Preschool Children Lois A. Maiman, Ph.D.
ZO1 HD 2107-04 PRJT. COMPLETED (10/1/94-8/1/95)	Children and Adolescent Trial of Cardiovascular Health (CATCH) Bruce G. Simons-Morton, Ed.D., M.P.H.
ZO1 HD 2108-04 PRJT. COMPLETED (10/1/94-7/1/95)	Afro-American Adolescent Girls Growth and Development Bruce G. Simons-Morton, Ed.D., M.P.H.
ZO1 HD 2109-04 PRJT. COMPLETED (10/1/94-4/1/95)	How Much Physical Activity Do Children Obtain During Physical Education Classes? Bruce G. Simons-Morton, Ed.D., M.P.H.
ZO1 HD 2110-04	Prevention of Problem Behaviors Among Middle School Students Bruce G. Simons-Morton, Ed.D., M.P.H.
ZO1 HD 2114-03	MD/Family Partnership: Education in Asthma Self-Management Lois A. Maiman, Ph.D.
ZO1 HD 2115-03	Immunization Among D.C. Infants Bruce G. Simons-Morton, Ed.D., M.P.H.
ZO1 HD 2116-03	Preventing Pregnancies Among Adolescent Girls in the District of Columbia Bruce G. Simons-Morton, Ed.D., M.P.H.
ZO1 HD 2117-02 PROJ. COMPLETED (10/1/94-6/15/95)	Validity of the Physical Activity Interview and Caltrac Motion Sensor Bruce G. Simons-Morton, Ed.D., M.P.H.
ZO1 HD 2118-01 PROJ. NEVER INITIATED-(CANCEL)	Parent-Child Conflict During the Transition to Middle School Barbara Radziszewska, Ph.D.; Denise Haynie, Ph.D.
ZO1 HD 2119-02	Parenting Style and Adolescent Substance Use and Depressive Symptoms Barbara Radziszewska, Ph.D.
ZO1 HD 2120-01 (5/2-9/30/95)	A Survey of Parent Intervention Strategies to Prevent Adolescent Alcohol Use Bruce G. Simons-Morton, Ed.D., M.P.H.
ZO1 HD 2121-01 (5/1-9/30/95)	Parent-Child Interaction and Asthma Self-Management Lois A. Maiman, Ph.D.
ZO1 HD 2122-01 (6/1-9/30/95)	Perceptions of Parents Regarding the Onset of Sexual Intercourse Among Youth Aria D. Crump, Sc.D.

ZO1 HD 2123-99

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
ZO1-HD-01600-11

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biochemical Basis of T Cell Activation

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: L.E. Samelson, Chief, SLS, CBMB
Others: J. Donovan, IRTA, CBMB
R. Guitian, Pre-IRTA, CBMB
N. Isakov, Adjunct Scientist, CBMB
Y. Ota, Visiting Fellow, CBMB
R.L. Wange, IRTA, CBMB

COOPERATING UNITS (if any)

R.N. Germain and D.I. Cohen, Laboratory of Immunology, NIAID; R. Aebersold and J. Watts, University of Washington; W. Langdon, University of W. Australia

LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Section on Lymphocyte Signaling

INSTITUTE AND LOCATION

NICHD, NIH

TOTAL STAFF YEARS:

6.0

PROFESSIONAL:

5.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Engagement of the multicomponent antigen receptor in T cells (TCR) results in rapid activation of a protein tyrosine kinase pathway. A major TCR-associated protein tyrosine kinase is ZAP-70, a protein that binds to the activated TCR. Under conditions of TCR crosslinking with anti-receptor antibodies, the ZAP-70 bound to the TCR is itself tyrosine phosphorylated and activated. Studies using T cell clones responding to peptide antigens demonstrate that ZAP-70 activation also occurs in that setting. For a given T cell clone, slight alteration of the peptide antigen or the major histocompatibility-encoded presenting molecule can result in partial activation or inhibition. Under these conditions, the TCR is partially tyrosine phosphorylated, and though the ZAP-70 molecule is bound to the TCR, it is neither tyrosine phosphorylated nor active. These studies have great relevance for understanding immunological tolerance as well as T cell activation. Moreover they demonstrate that regulation of ZAP-70 activity is at the level of phosphorylation as well as TCR binding. We have determined the sites of tyrosine phosphorylation of the ZAP-70 kinase. Using site-directed mutagenesis we have mutated two critical tyrosine residues in the kinase domain of ZAP-70, and have demonstrated that one of these sites is required for activation of the enzyme. These studies enhance our understanding of the mechanism of activation of this enzyme. Additional studies focus on a substrate of tyrosine kinases in T cells, p120^{cb1}, a proto-oncogene, which may be involved in the ras pathway in T cells. Recent studies demonstrate that this protein can be found in a complex with the linker protein Grb2. The amount of Grb2-p120^{cb1} complex is regulated by TCR activation. Studies on the dysregulation of tyrosine phosphorylation pathways seen with HIV infection have demonstrated that the tyrosine kinases Lck and Fyn, but not ZAP-70 are activated by HIV gp120 binding to CD4.

BIOCHEMICAL BASIS OF T CELL ACTIVATIONProject DescriptionObjectives:

The T cell antigen receptor (TCR) is a multicomponent structure that is coupled to several signal transduction pathways. Our earlier studies have demonstrated that receptor occupancy results in activation of tyrosine and serine kinases. Our current focus is the complete characterization of the protein tyrosine kinase pathway. The major goal of the unit is to identify and characterize the responsible kinase(s) and major substrates of these kinases. Only by this means will we be able to understand the function of this pathway and the manner by which it is activated and regulated.

Methods Employed:

Our studies of T cell activation use antigen specific murine T cell hybridomas, human T cell tumors, normal human peripheral T cells and murine thymocytes. Activation is induced either by specific antigen or stimulatory monoclonal antibodies. Analysis of signal transduction mechanisms depends on metabolic radiolabeling, detergent solubilization, immunoprecipitation and polyacrylamide gel electrophoresis. Phosphopeptide mapping has been performed to define sites of protein phosphorylation. Immunoblotting is used to detect antigen receptor subunits and tyrosine phosphorylated proteins. Immune complex kinase assays are used to determine protein tyrosine kinase activity in vitro. Protein tyrosine kinase domains are expressed in bacteria in order to obtain sufficient material for binding studies. Kinase protein is prepared using baculovirus expression systems. Phosphopeptides of defined sequence are synthesized to order. Kinase substrates have been purified by immunoaffinity chromatography, amino acid sequence has been obtained, and the cDNA encoding a protein tyrosine kinase substrate has been cloned. Mutated forms of this substrate have been generated using standard site-directed techniques. Eukaryotic expression vectors have been used to enable expression of substrates in T cells. Flow cytometry is used to characterized cell cycle progression.

The T Cell Receptor Tyrosine Kinase(s):

Identifying the kinase(s) responsible for tyrosine phosphorylation of the TCR and the other cellular substrates has been a major goal of this Section for many years. A critical tyrosine kinase activated by the T cell receptor is the ZAP-70 protein, which is tyrosine phosphorylated and associates with the TCR upon activation. Interesting features of this protein are

the tandem SH2 domains adjacent to the kinase domain. SH2 domains have been shown to bind proteins containing phosphotyrosine in other systems. In earlier studies we demonstrated that these two SH2 modules are required for binding to tyrosine phosphorylated TCR subunits. Well defined motifs in the tails of the CD3 and TCR ζ chains, the immunoreceptor tyrosine based activation motifs (ITAM), become tyrosine phosphorylated upon TCR engagement, and serve as the sites to which the tandem SH2 domains bind.

A series of experiments using normal T cell clones revealed that ZAP-70 binding to the TCR ITAMs is insufficient for enzyme activation. T cell clones can be activated to produce various lymphokines and proliferate following TCR engagement by defined peptides and major histocompatibility complex (MHC) molecules. However over the past several years it has become clear that slight alterations in this complex ligand, either by subtle changes in the peptide or the MHC molecule, can result in an altered response. Partial activation in which only certain lymphokines are produced or complete inhibition can occur. These variant ligands serve as models for ligands that normally might regulate T cell development in the thymus, induce anergy or determine the response to pathogens. Our studies using normal clones began with the demonstration that a ligand that fully activates the T cells results in TCR tyrosine phosphorylation, ZAP-70 binding and activation with tyrosine phosphorylation of intracellular substrates. These results were similar to that seen using the Jurkat T cell tumor line and TCR crosslinking by antibody. The response of these T cell clones to altered ligand, however, was dramatically different. An unusual form of TCR tyrosine phosphorylation was detected in which only partial TCR chain tyrosine phosphorylation occurred. ZAP-70 binding to this subunit was detected, but this molecule was not tyrosine phosphorylated and was not active. Only minimal tyrosine phosphorylation of intracellular substrates was detected. These studies are relevant for understanding T cell activation during thymic development and induction of anergy as mentioned. They also show that binding of ZAP-70 to the TCR is insufficient for its activation, and focus attention on the regulation of the kinase by phosphorylation.

With collaborators in Vancouver and Seattle we previously mapped the sites of tyrosine phosphorylation of the ZAP-70 kinase. Using a baculoviral expression system we obtained large quantities of the kinase. Sites of autophosphorylation and sites that became phosphorylated after addition of recombinant Lck protein were determined by mass spectrometric analysis of ZAP-70 tryptic peptides. Upon determination of these sites of phosphorylation in vitro, it was then possible to show that tyrosine residues Y292, Y492 and Y493 become phosphorylated after TCR engagement of intact cells. Site directed mutagenesis using ZAP-70 cDNA was used to mutate residues Y492 and Y493 to phenylalanine. These

constructs were then expressed in COS cells alone or with Lck constructs and ZAP-70 activity was then assayed by in vitro kinase reactions. Native ZAP-70 expressed in COS cells has a basal level of activity that is markedly increased by co-expression of active Lck. Mutation at Y493 results in an enzyme with the basal level of activity but no enhanced response to co-expressed Lck. Phosphorylation at this site by Lck is likely a critical first step in activation of the enzyme. The mutation of Y492 to phenylalanine gave the unexpected result of leading to a four-fold increase in kinase activity, which was increased even more with Lck co-expression. It is possible that tyrosine phosphorylation at this residue is normally inhibitory. However we favor the model that Y492 when non-phosphorylated along with Y493 interact with critical residues in the kinase domain to maintain the enzyme in an inactive state. Molecular modeling studies support this hypothesis. The Y492F mutation would then result in disinhibition leading to an active kinase.

The Tyrosine Phosphorylation Pathway in T cells:

In previous studies we have demonstrated that activation of the TCR results in phosphorylation of the TCR ζ chain and multiple intracellular substrates. The identity of the other substrates that are tyrosine phosphorylated upon TCR activation has been a major question for this Section for some time. One substrate of TCR-mediated tyrosine kinase activation is the protooncogene p120^{cb1}. In its oncogenic form, expressed as a fusion protein with a retroviral gag sequence the p100^{v-cb1} protein transforms pre-B cells and is expressed in the nucleus. The p120^{cb1} protein is non-transforming and is entirely cytoplasmic. It is rapidly tyrosine phosphorylated upon T cell activation. It has been shown to bind a number of critical intracellular signaling proteins. We showed earlier that the SH3 domains of one such protein, the linker molecule Grb2 binds to p120^{cb1} in vitro. More recently we have demonstrated that a Grb2-p120^{cb1} complex can be found in the cell. Binding here too is mediated by the Grb2 N-terminal SH3 domain. Interestingly this complex is regulated by TCR engagement as the complex dissociates upon TCR engagement. Studies to determine the function of p120^{cb1} are in progress.

Abnormal tyrosine phosphorylation of intracellular substrates might reflect inappropriate activation of intracellular signalling pathways. In collaboration with Dr. D.I. Cohen, LIR, NIAID, we have determined that such abnormal tyrosine phosphorylations occur in T cells after infection with HIV. We have discovered that one of the prominent tyrosine phosphorylated substrates is p34cdc2, a regulator of mitosis at the G2/M boundary. Cells dying of HIV-induced killing are arrested at this checkpoint. We have recently demonstrated other abnormalities of the tyrosine phosphorylation pathway. Engagement of the CD4 molecule by HIV gp120 results in activation of the Lck and Fyn

protein tyrosine kinases without either TCR ζ chain phosphorylation or ZAP-70 activation. Activation of the two src-family kinases is independent of TCR activation. Whether this pattern of activation of these kinases is coupled to HIV-induced cell death is under investigation.

Publications

Isakov N, Wange RL, Burgess WH, Watts JD, Aebersold R, and Samelson LE. ZAP-70 binding specificity to T cell receptor tyrosine-based activation motifs: The tandem SH2 domains of ZAP-70 bind distinct TAMs with varying affinity. J Exp Med 1995;181:375-380.

Kolesnitchenko V, Wahl LM, Sunila I, Tian H, Tani Y, Hartmann D-P, Cossman J, Raffeld M, Orenstein J, Samelson LE, and Cohen DI. HIV-1 envelope-initiated G2 phase programmed cell death. Proc Natl Acad Sci USA 1995 (In press).

Madrenas J, Wange RL, Wang JL, Isakov N, Samelson LE, and Germain RN. ζ phosphorylation without ZAP-70 activation induced by T cell receptor antagonists or partial agonists. Science 1995;267:515-518.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01602-11

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Regulation of Intracellular Iron Metabolism

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LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Section on Human Iron Metabolism

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:

8.55

PROFESSIONAL:

7.75

OTHER:

.8

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project is aimed at understanding the molecular basis of intracellular iron metabolism. The cis and trans elements mediating the iron-dependent alterations in abundance of ferritin and the transferrin receptor have been identified and characterized in previous years in this laboratory. Iron-responsive elements (IREs) are RNA stem-loops found in the 5' end of ferritin mRNA and the 3' end of transferrin receptor mRNA. We have cloned, expressed, and characterized two essential iron-sensing proteins, Iron Regulatory Protein 1 (IRP1) and Iron Regulatory Protein 2 (IRP2), formerly referred to as iron-responsive element binding proteins (IRE-BPs). IRPs bind IRE's when iron levels are depleted, resulting in the inhibition of translation of ferritin mRNA and prolongation of the half-life of the transferrin receptor mRNA. IRP1 is an iron-sulfur protein related to mitochondrial aconitase, a Krebs cycle enzyme, and it functions as a cytosolic aconitase in cells that are iron replete. In cells that are iron-depleted, IRP2 binds IREs with high affinity. Regulation of RNA binding activity involves a transition from a form of IRP1 in which a [4Fe-4S] cluster is bound, to a form that loses both iron and aconitase activity. The [4Fe-4S] containing protein does not bind IREs. Controlled degradation of the iron-sulfur cluster reveals that the physiologically relevant form of the RNA binding protein in iron-depleted cells is apoprotein. The status of the cluster appears to be the key to determining whether the protein will bind RNA. Little is known about the assembly and disassembly of iron-sulfur clusters in mammalian cells. In recent studies, we have identified mammalian genes homologous to genes that are implicated in bacterial iron-sulfur cluster assembly. Unlike IRP1, IRP2 is actively degraded in cells that are iron-replete. An additional exon present in IRP2 determines whether IRP2 is subject to degradation. This 73 amino acid exon is sufficient to confer upon IRP1 an iron-dependent degradation phenotype. Mutagenesis of cysteines within the 73 amino acid domain reveals that cysteines are required for degradation, and treatment of cells with proteasome inhibitors reveals that the proteasome is required for degradation. In order to approach questions about the physiology of iron metabolism, IRP1 has been "knocked out" in mice, using homologous recombination in embryonic cell lines. There is no obvious phenotype associated with loss of IRP1 function, and we speculate that both IRPs must be absent in order to see perturbations in iron metabolism. Accordingly, creation of IRP2 "knockouts" and double "knockouts" are underway.

REGULATION OF INTRACELLULAR IRON

Project description

Objectives

The main objective of this project is to understand mammalian iron metabolism. We have cloned and characterized through site-directed mutagenesis the two major regulatory proteins involved in sensing iron levels, IRP1 and IRP2. In order to better understand their mode of regulation, iron-sulfur cluster assembly and disassembly is being studied *in vitro*. Pulse-chase studies have been used to define the iron-dependent degradation of IRP2 and mutagenesis is being utilized to better characterize the degradation signals. Over-expressed and purified apoprotein is being used to attempt to create crystals of the apoprotein and co-crystals with the RNA ligand. Homologous recombination and electroporation of embryonic stem cells has been used to create mice that are heterozygous or homozygous for deficiency of IRP1 and/or IRP2.

Methods Employed

Standard techniques of molecular biology are employed including cloning, site-directed mutagenesis using 2 step PCR methodology, and transient and stable transfection. Protein chemistry techniques include one and two-dimensional electrophoresis, immunoprecipitation, biosynthetic labeling, and FPLC. Gel-retardation and resolution of complexes is performed on native gels, and *in vitro* translation systems are employed.

Major Findings

Expression of a constitutive mutant of iron regulatory protein 1 abolishes iron homeostasis in mammalian cells

Iron regulatory proteins (IRPs) are iron-sensing proteins that bind to RNA stem-loop sequences known as iron-responsive elements (IREs) when cells are depleted of iron. Although IRPs have been previously shown to bind to IREs derived from ferritin and TfR mRNAs *in vitro*, there has not been a direct demonstration of the impact of a recombinant IRP on the expression of endogenous IRE-containing transcripts. Modulation of binding of iron regulatory protein 1 (IRP1) to IREs occurs through the assembly and disassembly of an iron-sulfur cluster and consequent modification of the RNA binding site. Mutation of each of the cysteines thought to ligate the iron-sulfur cluster of IRP1 produces a form of the protein that constitutively binds RNA. We evaluated the impact of expression of C437S, a mutant of IRP1 which binds IREs regardless of cellular iron status, on the regulation of biosynthesis of ferritin and the transferrin receptor. Expression of the mutant IRP1 prevented the normal regulation of ferritin and transferrin receptor expression. Cells continued to synthesize and express high amounts of TfR even when cells were iron-replete, while synthesis of ferritin remained repressed when cells were iron-replete. Thus, a single mutant IRP can prevent the usual homeostatic changes in synthesis of the transferrin receptor and ferritin. Cells expressing the mutant protein would therefore be predicted to be unable to defend against iron-overload. Preliminary results have shown that cells are killed by treatment with iron when C437S is expressed, and we are working towards creation of a tissue culture model system for the study of iron toxicity (DeRusso et al., 1995).

IRP2 is regulated by protein degradation in iron-replete cells

Binding studies have shown that IRP2 binds to consensus IREs with high affinity and specificity similar to IRP1. Whereas the RNA binding activity of IRP1 is regulated by the reversible assembly and disassembly of an iron-sulfur cluster in an otherwise stable protein, absolute levels of IRP2 are markedly decreased by iron treatment in several different cell types and the decrease in protein levels can be accounted for by an increase in the rate of degradation of IRP2 (Samaniego et al., 1994).

A sequence unique to IRP2 is required for rapid iron-dependent degradation

IRP1 and IRP2 are highly homologous proteins with an overall sequence identity of 58%. A major distinguishing feature between IRP1 and IRP2 is the presence of an insertion of 73 amino acids in domain 1 of IRP2. Cloning of the genomic fragment corresponding to this sequence has revealed that the 73 amino acids are encoded by a single exon. When the 73 amino acid insertion was excised by site directed mutagenesis from IRP2, levels of the IRP2 exon deletion mutant (IRP2-73) were no longer markedly decreased after treatment with iron. The IRE binding affinity remained high, with an estimated K_d of 10-50 pM, and specificity for the consensus IRE was unchanged as judged by competition assays with unlabeled IREs and unrelated stem-loops. These results indicated that the fundamental tertiary structure of IRP2 was unchanged by excision of this exon sequence.

In order to verify that the IRP2 exon deletion mutant (IRP2-73) accumulated because of a decrease in the rate of degradation, as has been shown for wild type IRP2, pulse chase experiments were performed. The rate of degradation of IRP2 wild type in iron-treated cells was markedly faster than the rate of degradation of the IRP2 specific exon deletion mutant ($t_{1/2}$ of 3-6 h vs. 24h), thus supporting the hypothesis that the IRP2 specific exon may contain degradation signals which are activated by iron.

Insertion of the IRP2 specific exon into IRP1 results in iron-dependent degradation of recombinant IRP1

To assess whether the 73 amino acid IRP2-specific exon contained sufficient information to direct iron regulated degradation of IRP2, the sequence was cloned into IRP1 at a homologous position. When assayed in a pulse chase experiment in the presence of iron, the IRP1/IRP2 specific exon chimeric protein (IRP1+73) was much more rapidly degraded than wild-type IRP1 and the degradation rate was comparable to that of wild type IRP2 ($t_{1/2}$ of approximately 3-6 h). Thus, the IRP2 specific exon conferred the capacity to be degraded at a rate similar to that seen in intact IRP2 upon IRP1.

The iron-dependent degradation signal of IRP2 requires the participation of cysteines in the IRP2 specific exon

Cysteines are known to be critical in ligation of the [4Fe-4S] cluster of IRP1. In that setting, the iron-sulfur cluster is ligated by three cysteines, C437, C503, and C506, and it is the presence of a fully assembled [4Fe-4S] cluster that prevents RNA binding. In addition, mutation of C437 to serine (C437S) or of all three cysteines 437, 503, 506 to serines results in expression of a mutant protein in which IRE binding is no longer regulated and in which the mutant protein is permanently fixed in the IRE binding mode. In order to assess whether the corresponding cysteines were required for iron-dependent degradation of IRP2 or the IRP1/IRP2 specific exon chimeric protein (IRP1+73), a series of chimeric proteins containing cysteine mutations were assessed. Mutation of one or several of the cysteines required for cluster ligation of IRP1 did not interfere with iron-induced degradation of chimeric IRP1/IRP2 specific exon chimeric constructs. Thus, the iron sensing capability of the IRP2 insert does not depend on participation of cysteines homologous to previously identified cysteine ligands of the iron-sulfur cluster.

The presence of five cysteines in the IRP2 specific exon, with the spacing C (X₃₀)C(X₅)C(X₃)C(X₂₃)C offered another set of cysteines which could be assessed for their role in iron-sensing. The close physical grouping of the three middle cysteines of the exon is noteworthy and we questioned whether the cysteines might be acting in concert to sense iron levels. In order to test whether cysteines from the IRP2 specific exon were important in degradation, the degradation rate of the chimeric protein IRP1/IRP2 specific exon (IRP1+73) was compared to the degradation rate of a similar construct which differed only in that the middle three cysteines, C168, C174 and C178, of the IRP2 specific domain were simultaneously converted to serines by site-directed mutagenesis (Exon 3C-S). When the three cysteines were mutated to serines, the IRP2 specific exon could no longer transfer the capacity for rapid iron-dependent degradation to IRP1. A pulse

chase experiment revealed that the exon 3C-S mutant was stable, with an estimated $t_{1/2}$ of 24 h. Gel-retardation assays of all mutant chimeras indicated that IRE binding activity was intact and that the tertiary structure was therefore fundamentally intact. Thus it appears that cysteines are critical to presentation of the signal for iron-dependent rapid degradation, possibly because the cysteines may be involved in direct ligation of iron or an iron-sulfur cluster.

Inhibition of proteasome function in vivo prevents iron dependent degradation of IRP2

In order to gain insight into the mechanism of degradation, iron-replete cells were treated with a variety of reagents known to interfere with various modes of proteolysis in cells. The peptide aldehyde MG132, a potent inhibitor of the 20 S subunit of the proteasome, interfered with degradation of IRP2 when cells were treated with iron. To further confirm the role of the proteasome in degradation of IRP2, a more specific inhibitor of proteasome function, lactacystin, was used. Lactacystin inhibits proteasome function by covalently binding to the amino terminal threonine of the mammalian proteasome subunit X. The effects of lactacystin in the cell are more specific for the proteasome than are those of MG132 and we found that lactacystin inhibits degradation of IRP2 at a level comparable to MG132. To confirm that a decrease in degradation rather than a change in the level of synthesis was the cause of increased protein levels, pulse chase experiments were performed which confirmed that degradation was inhibited. Thus, degradation of IRP2 is most likely mediated by the proteasome (Iwai et al., 1995).

Translational Repressor Activity is Equivalent and is Quantitatively Predicted by in vitro RNA Binding for Two IRE Binding Proteins, IRP1 and IRP2

The existence of two IRE binding proteins which are regulated by different mechanisms has increased the complexity of the IRP regulatory system. Thus far, there have been no descriptions of cell types in which one form of IRP is expressed while the other is not. It remains unclear why there are two IRE binding proteins. One possibility would be that the two IRPs respond to different stimuli. Another possibility is that the targets of the two proteins differ. Though we have previously shown using gel retardation studies that the two IRPs bind with equal affinity to isolated IRE motifs derived from ferritin, eALA synthase, and the transferrin receptor mRNAs, these studies do not necessarily mean that in the setting of the intact transcript that the two proteins bind with equal affinity to target IREs, as context and the potential for protein-protein interactions may modify binding affinity *in vivo*.

We utilized purified recombinant IRP1 and IRP2 and compared the ability of each to repress translation of ferritin *in vitro*. We showed that equal molar amounts of IRP1 and IRP2 bind isolated IREs equally, and furthermore that equal binding in the gel retardation assay predicted equal impact on translation of a target mRNA (Kim et al., 1995).

IRP1 is not indispensable in mice

Homologous recombination has been used to "knockout" IRP1 in mice. Heterozygotes for the "knockout of IRP2 have been created. Mice lacking IRP1 only are healthy, and iron uptake and metabolism appear to be normal. Breeding of the mice should enable us to assess the phenotype of mice lacking the gene products of both IRP1 and IRP2.

Publications

DeRusso PA, Philpott CC, Iwai K, Mostowski HS, Klausner RD, and Rouault TA. Expression of a constitutive mutant of iron regulatory protein 1 abolishes iron homeostasis in mammalian cells. JBC 1995;26:15451-15454.

Iwai K, Klausner RD, and Rouault TA. Requirements for iron regulated degradation of the RNA binding protein, Iron Regulatory Protein 2 (IRP2). EMBO J 1995(In press).

Jaffrey SR, Cohen NA, Rouault TA, Klausner RD, and Solomon SH. The iron-responsive element binding protein: A novel target for synaptic actions of nitric oxide. Proc Natl Acad Sci USA 1994;91:12994-12998.

Kim HY, Klausner RD, and Rouault TA. Translational repressor activity is equivalent and is quantitatively predicted by in vitro RNA binding for two iron-responsive element binding proteins, IRP1 and IRP2. JBC 1995;4983-4986.

Rouault TA, Klausner RD, and Harford JB. Translational control of ferritin. In: J Hershey et al, eds. Translation Regulation. Cold Spring Harbor: Cold Spring Harbor Press 1995(In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01606-07

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Biology of Early Organelles of the Secretory Pathway

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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COOPERATING UNITS (if any)

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LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Section on Organelle Receptor Structure and Function

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:

4.25

PROFESSIONAL:

3.25

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The central vacuolar system of eukaryotic cells is an interlocking membrane system composed of distinct organelles (including ER, Golgi apparatus, secretory vesicles, secretory granules, plasma membrane, endosomes and lysosomes) and selective transport pathways. Proper functioning of this system is vital for regulating surface expression and secretion of proteins. Using the fungal metabolite brefeldin A (BFA), which dramatically alters both the distribution and flow of membrane through the central vacuolar system, this group has focused on how organelles maintain their structure and identity, and how membrane trafficking between organelles is regulated. These questions have been directed to three different areas: (1) Understanding the biochemical basis of BFA action. We have found that BFA appears to affect the Golgi apparatus by acting on specific membrane targets that regulate the assembly of cytosolic coat proteins onto the cytoplasmic face of this organelle. Altered interaction of the coats with the organelle leads to organelle disassembly, tubule formation and mixing of organelle components within a defined "homotypic" membrane system. The biochemistry of two BFA sensitive coat proteins, α -COP and ARF are under investigation. (2) The biology and biochemistry of ARF family proteins. The recognition that the activation of the family of small GTP binding proteins called ARF's is most likely the target for BFA has led us to an extensive study of the biochemistry and cell biology of individual ARF family proteins using *in vitro* reconstitution of biochemical assays, mutagenesis and expression of wild type and mutant ARF proteins in cells. (3) Characterization of the distribution and flow of membrane within the vacuolar system. We have found that BFA causes the various compartments of the vacuolar system to collapse into new steady states which produce isolated but functioning new organelle units. Traffic within but not between these units continues in the presence of BFA. In summary, our studies with BFA provide new insights about the properties of sets of organelle-specific coat proteins and presents a framework for relating the biochemical regulation of membrane transport to the structure and maintenance of organelles. We have continued studies on the mechanisms of retention of proteins within the early organelles of the vacuolar system.

THE BIOLOGY OF EARLY ORGANELLES OF THE SECRETORY PATHWAY

Objectives

Over the past year our efforts have focused on the mechanisms regulating cell shape and membrane traffic at the plasma membrane. We have pursued three projects in this area:

- 1) The role of the ARF6 GTPase in the control of membrane traffic and membrane modeling at the plasma membrane.
- 2) Endocytosis and plasma membrane protein turnover in the budding yeast *Saccharomyces cerevisiae*.
- 3) Cell shape and cell polarity in the fission yeast *Schizosaccharomyces pombe*.

ARF6: A GTPase implicated in plasma membrane modeling, actin rearrangements and formation of pseudopodia.

Over the past several years, we have been studying the ARF (ADP-ribosylation factor) family of GTP binding proteins and their role in membrane traffic. We had showed that the ARF1 and ARF3 proteins function to regulate transport along the secretory pathway through the assembly of cytosolic coat proteins with membranes of the Golgi apparatus. There are at least three other distinct ARF family members, ubiquitously expressed in all cells. To begin to define the function of the other ARF proteins, we have analyzed the localization and cellular phenotypes of transiently expressed, epitope-tagged ARF proteins and their mutants in mammalian cells. For ARF6, we found that in cells overexpressing the wild type protein, the ARF6 protein is associated with the plasma and endosomal membranes, and that cellular morphology was normal. A mutant ARF6 predicted to be defective in GTP binding (T27N), and hence predominantly in the GDP state, is associated exclusively with endosomes, whereas a GTP hydrolysis-defective mutant (Q67L) is confined to the plasma membrane. The morphology of cells expressing these mutant forms of ARF6 is altered: cells expressing T27N exhibit an accumulation of tubular endosomal structures and cells expressing the Q67L GTP-active mutant exhibit an elaboration of plasma membrane folds at peripheral edges of these cells.

In order to better define the ARF6 function, we tested various pharmacologic agents for the ability to shift the distribution of ARF6 and the phenotype of cells overexpressing the wild type protein into that of cells overexpressing the active, GTP-hydrolysis defective mutant (Q67L). We found that treatment of HeLa cells transfected with wild type, epitope-tagged ARF6 with the G protein activator, aluminum fluoride (AlF) resulted in a redistribution of both ARF6 and actin to discrete sites on the plasma membrane which became increasingly protrusive over time. These structures resemble pseudopodia in that they contain actin, membranous folds and are dynamic structures forming and disassembling over a period of minutes. Immuno-EM showed that these pseudopodia contained numerous membranous folds similar to those in cells expressing the GTP-hydrolysis mutant ARF6/Q67L. The effects of AlF were reversible and specific to cells transfected with wild type

ARF6; pseudopodia were not observed in cells transfected with non-myristoylated ARF6/G2A, GTP-binding defective ARF6/T27N, wild type ARF1, or in untransfected cells. Thus, it appears that AIF treatment results in the reversible "activation" of ARF6, allowing us to better characterize the ARF6 effector function, the extreme of which is the Q67L-induced extensive membranous folds. The AIF-induced pseudopodia in ARF6-overexpressing cells were distinct from actin rearrangements observed in cells overexpressing the Rac1 protein, a GTPase reported to be involved in membrane ruffling. These cells formed lamellopodia when treated with AIF, and mimicked the phenotype observed in cells expressing the Q61L, GTP active Rac1 mutant.

In addition to actin recruitment and rearrangements, other actin-associated proteins including gelsolin, focal adhesion kinase (FAK), and cortactin, were also recruited into the pseudopodia. Coupled to the dynamic pseudopodia formation is an apparent increase in the amount of membrane turnover via fluid phase pinocytosis. During AIF-induced pseudopod formation, fluid phase markers were rapidly and transiently taken up into macropinosomes within the pseudopods. This striking localized turnover of plasma membrane however, did not appear to significantly alter either transferrin uptake into the cell nor the distribution of clathrin AP-2 complexes along the plasma membrane. These results suggest that ARF6, like the Rho-related GTPases, can regulate the actin cytoskeleton and induce changes in plasma membrane architecture. We are currently investigating the requirements for the AIF induced pseudopod formation and find that arachidonic acid metabolism, specifically leukotriene synthesis, is involved in this process.

Surface Protein Turnover in *Saccharomyces cerevisiae*

The research over the past year has addressed the means by which *S. cerevisiae* achieves copper homeostasis via regulation of turnover of its plasma membrane copper transporter Ctr1p. Since surface protein turnover is expected to be mediated by internalization and delivery to the vacuole where hydrolases reside, this system should provide a setting for the study of endocytosis and intracellular trafficking in yeast.

The Ctr1p is a copper transporter in the plasma membrane of *S. cerevisiae* that provides the limiting step for high affinity copper uptake into the cell. Maintenance of copper homeostasis is thus likely to involve modulation of cell surface levels of Ctr1p. We have demonstrated that Ctr1p is a stable protein under conditions of low copper but that addition of high copper (micromolar levels) triggers degradation of cell surface Ctr1p. This degradation is seen whether Ctr1p synthesis and expression is controlled by its own copper-responsive promoter, or by a heterologous galactose-inducible promoter.

We then addressed the mechanism by which copper-triggered Ctr1p degradation was occurring. Contrary to expectation, we find that internalization and delivery to the vacuole does not appear to be the principal mechanism that is responsible for this degradation. Copper dependent degradation of Ctr1p takes place in a number of mutant strains defective in endocytosis and vacuolar hydrolysis. Our results suggest that we may have uncovered a novel pathway of surface proteolysis in yeast that regulates surface expression of proteins.

Further experiments under conditions of lowered temperature indicate, however, that endocytosis and delivery to the vacuole may play a minor role in copper-dependent degradation of Ctr1p. We have also found that brief exposure to copper induces a shift of Ctr1p into a Triton-insoluble fraction, implying that a change in the physical state of Ctr1p may be involved in copper-dependent degradation.

Cell Shape and Cell Polarity in Fission Yeast

We want to understand how cells control cell morphogenesis and cell polarity. Cells possess specialized shapes that accommodate their function. One aspect of cell morphogenesis is cell polarity, the ability of the cell to establish spatially distinct domains that fulfill distinct functions. This phenomenon is widespread in biology and can be identified in both prokaryotes and eukaryotes. We have chosen fission yeast *Schizosaccharomyces pombe* as a model organism to start studying cell shape and cell polarity because it is an unicellular eukaryote with a well-defined cylindrical rod shape. The length of the cell increases as the cell grows, and this cell growth is polarized; it only occurs at the tips of the cells and not at the cylindrical middle part. In other words, new cell wall material is incorporated specifically at the cell tips, probably involving the secretory pathway. The main focus of our study in *Schiz. pombe* is to understand how cells establish, maintain, and control polarized cell growth.

Our strategy for identifying new proteins and pathways that regulate cell morphogenesis and polarity consists of the generation of a panel of cell shape mutants of *Schiz. pombe* that are round, in contrast to wild-type cells which are rod-shaped. Wild-type cells were treated with the mutagen ethyl methane sulfonate and rounded mutants were isolated visually. Those mutants with a single gene defect were analyzed further.

1) Characterization of J3 cell shape mutant:

In our first cloning attempt, we chose the recessive mutant J3 because its cell shape shows a remarkable temperature-dependence. At the standard growth temperature, the cells are round and pear-shaped. However, its cell shape phenotype changes upon changes of the growth temperature. At a lower temperature, J3 cells display a wild-type cylindrical rod-shape. This property allows us to induce the mutant phenotype through a simple temperature change. At a higher, nonpermissive temperature, the cells showed an increase in the amount of membranes of the secretory pathway over control cells, with an apparent abundance of endoplasmic reticulum and Golgi apparatus. Also, the septa and cell walls were several times thicker than those of control cells. Eventually, the mutant cells lyse at the nonpermissive temperature.

2) Efforts to clone the complementing gene and multi-copy suppressor genes.

We have started experiments to isolate the gene mutated in J3 cells. Because a standard gene rescue experiment did not allow identification of the mutant gene (see next paragraph), we are following a positional cloning approach. The J3 locus was found to map to a region on chromosome III and one

cosmid clone that covered this chromosomal region was found to complement the J3 temperature-sensitive phenotype. Experiments to subclone and sequence the complementing gene are in progress.

In standard gene rescue experiments, a wild-type genomic library was introduced into the recessive mutant and transformants with a wild-type phenotype were selected. We isolated six distinct clones which represent extragenic suppressors for mutant J3. To determine the nature of these suppressor clones, we sequenced four of the six genomic clones. Interestingly, all four clones contained a partial open reading frame (ORF). One clone showed sequence similarity to a *Sacc. cerevisiae* cell wall enzyme β -1,3-glucanase, a second showed sequence similarity to the *Sacc. cerevisiae* cell surface protein, while the two other clones bore no similarity to known proteins. All four ORFs were partial, because none of the genomic inserts contained a termination codon. When the protein sequences were analyzed for the presence of a shared motif that might be responsible for the multi-copy suppression, we observed an amino-terminal hydrophobic signal sequence in all four ORFs, indicating that these polypeptides enter the secretory pathway.

The finding that overexpression of secretory polypeptides might correct the defect of the J3 mutant is significant, considering the observation that the mutation causes changes in the secretory pathway, that, in turn, might be responsible for the changes in cell shape.

Publications

Donaldson JG, Radhakrishna H, and Peters PJ. The ARF GTPases: Defining roles in membrane traffic and organelle structure. Cold Spring Harbor Symposium on Quantitative Biology, Vol 60, 1995(In press).

Peters PJ, Hsu VW, Ooi CE, Finazzi D, Teal SB, Oorschot V, Donaldson JG and Klausner RD. Overexpression of wild-type and mutant ARF1 and ARF6: Distinct perturbations of nonoverlapping membrane compartments. J Cell Biol 1995;128:1003-1017.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01607-05

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Protein Trafficking in the Secretory Pathway

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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M.C. Fournier, Visiting Fellow, CBMB

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COOPERATING UNITS (if any)

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LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Unit on Intracellular Protein Trafficking, Section on Organelle Receptor & Function

INSTITUTE AND LOCATION

NICHHD, Bethesda, MD

TOTAL STAFF YEARS:

6.8

PROFESSIONAL:

5.0

OTHER:

1.8

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This group investigates the molecular mechanisms that determine the intracellular localization and sorting of integral membrane proteins within the endocytic and secretory pathways. This past year, our work has been directed towards identifying and characterizing: (i) structural signals that specify localization and sorting of integral membrane proteins, (ii) proteins that recognize the sorting signals, and (iii) other components of the sorting machinery.

The nature of signals involved in sorting of integral membrane proteins was investigated using as models a molecule involved in antigen presentation, HLA-DM, and the endopeptidase, furin. HLA-DM is localized to an endosomal/lysosomal compartment where class II molecules of the major histocompatibility complex (MHC) encounter and bind antigenic peptides. Our studies showed that targeting of HLA-DM to this compartment is largely mediated by a tyrosine-based signal (YTPL) in the cytoplasmic domain of the β chain of the molecule. Furin is predominantly localized to the *trans*-Golgi network (TGN). Molecular dissection of furin demonstrated the existence of two targeting signals that contribute to the localization of furin to the TGN: a tyrosine-based signal (YKGL) and a novel acidic sequence (WQEECPDSEEDGRGER). This novel acidic signal was shown to be a site of phosphorylation by casein kinase II, suggesting a role for phosphorylation in regulating the trafficking of furin.

A search for proteins that bind to tyrosine-based signals resulted in the identification of the medium chains (μ_1 and μ_2) of clathrin-associated adaptor complexes (AP-1 and AP-2) as the signal-recognition components of the sorting machinery. This finding opened the way for a detailed characterization of the interaction of signals and recognition proteins at a molecular level.

PROTEIN TRAFFICKING IN THE SECRETORY PATHWAY

Project Description

Objectives:

This group investigates the molecular mechanisms that determine the localization and sorting of integral membrane proteins within the endocytic and secretory pathways. The main objectives of this project are the identification of signals involved in protein localization and sorting within the *trans*-Golgi network (TGN) and the endosomal/lysosomal system and of recognition molecules that bind to the signals.

Methods Employed

All projects in this group involved the use of recombinant DNA procedures, morphologic and biochemical techniques, and yeast genetic approaches. In order to search for sorting signals within several integral membrane proteins, we constructed mutant and chimeric proteins using the polymerase chain reaction (PCR) and other standard recombinant DNA procedures. Constructs were expressed in mammalian cells by either transient or stable transfection. The subcellular localization of the different protein constructs was determined by immunofluorescence microscopy, image analysis, and quantitative immunoelectron microscopy, and their biosynthesis, processing and degradation were studied by pulse-chase metabolic labeling and immunoprecipitation. The ability of signals to mediate internalization from the cell surface was examined using quantitative endocytosis assays. We also examined the phosphorylation and dephosphorylation of some of the sorting signals by labeling with ^{32}P -phosphate both *in vivo* and *in vitro*. Proteins that bind to sorting signals (recognition molecules) were identified using a yeast two-hybrid system and affinity purification with glutathione-S-transferase (GST) fusion proteins. The interaction of sorting signals with their recognition molecules was characterized using various *in vitro* binding assays and surface plasmon resonance spectroscopy. Complementary DNAs encoding the recognition molecules and related proteins were cloned using established recombinant DNA procedures.

Major Findings

Cytoplasmic Sorting Signals.

It is now well established that the localization of integral membrane proteins to specific compartments of the secretory pathway is mediated by information encoded within the structure of the proteins. In some cases, this information derives from a global physicochemical property of the proteins, such as a tendency to aggregate in certain cellular environments. In other cases, information is contained within short, linear arrays of amino acid residues that act as specific sorting ("address") signals. Our group has been particularly interested in the mechanisms that determine protein localization to compartments of the

peripheral secretory pathway, such as the TGN, lysosomes and endosomes, for which cytoplasmic sorting signals play a major role. The sorting signals are thought to interact with cytoplasmic receptor-like molecules that function as recognition components of the sorting machinery. The main objectives of our work this year have been the identification of sorting signals within proteins that are targeted to the TGN and the endosomal/lysosomal system and the search for the specific signal-recognition molecules.

A Tyrosine-based Signal Targets a Component of the Antigen Presentation Machinery, HLA-DM, to the Endosomal/Lysosomal System.

HLA-DM is a membrane-bound heterodimer ($\alpha\beta$) that promotes loading of antigenic peptides onto class II molecules of the major histocompatibility complex (MHC). In antigen presenting cells, HLA-DM is localized to a compartment of the endosomal/lysosomal system known as the MIIC (for MHC class II compartment).

We conducted a systematic analysis of sorting information within HLA-DM and found that the localization of HLA-DM to the MIIC was directed by a tyrosine-based signal (YTPL) within the cytoplasmic domain of the β chain of the molecule. This signal was active both as an internalization and lysosomal targeting signal, thus establishing a biogenetic relationship of the MIIC with the endosomal/lysosomal system.

Two Independent Targeting Signals Mediate Localization of the Endopeptidase, Furin, to the TGN.

Furin is a dibasic endopeptidase responsible for the proteolytic processing of numerous protein precursors, including pro-hormones and viral envelope glycoprotein precursors. Our previous studies had demonstrated that furin was predominantly localized to the TGN and that the cytoplasmic domain was both necessary and sufficient for TGN localization. This past year, we have performed a systematic analysis of targeting information within the furin molecule. Our studies have uncovered the existence of two targeting signals that contribute to the TGN localization of furin. The first signal is a typical tyrosine-based motif, YKGL. The second signal is distinct from other signals that have been described to date. It consists of a strongly hydrophilic sequence containing a cluster of acidic amino acid residues (WQEECPDSEDEGRGER). Both signals can mediate internalization from the cell surface, but only the acidic sequence is capable of conferring localization to the TGN *per se*. We also found that two serine residues within the acidic sequence are phosphorylated by casein kinase II *in vivo* and *in vitro*. This observation suggests a role for phosphorylation in regulating the localization and trafficking of furin.

Identification of the Medium Chains of Clathrin-associated Adaptor Complexes as the Recognition Molecules for Tyrosine-based Sorting Signals.

During the past year, we undertook several approaches to identify molecules that bind to tyrosine-based signals and thereby mediate their sorting functions. Using a yeast two-hybrid system, we found that the medium (μ) chains of clathrin-associated adaptor complexes

interact specifically with tyrosine-based signals. Both μ_1 (a component of TGN adaptors) and μ_2 (a component of plasma membrane adaptors) are capable of binding to various tyrosine based signals, such as those of the proteins TGN38 (YQRL), HLA-DM (YTPL), LAMP-1 (YQTI), CD68 (YQPL) and the transferrin receptor (YTRF). *In vitro* binding assays confirmed the results of the two-hybrid system and allowed a detailed characterization of the interactions. The identification of the μ_1 and μ_2 as the recognition molecules for tyrosine-based signals supports a role of clathrin coats and tyrosine-based signals in sorting at both the TGN and the plasma membrane.

In Vivo Studies of the Saturability of Sorting Processes Mediated by Tyrosine-Based Signals.

The existence of specific recognition molecules that bind to tyrosine-based sorting signals predicts that processes mediated by such signals should be saturable. We tested this prediction by overexpressing in transfected cells proteins that have tyrosine-based signals and analyzing the surface expression of other proteins that are normally targeted to lysosomes by virtue of tyrosine-based signals. We observed that such overexpression interfered with several processes dependent on tyrosine-based signals such as lysosomal targeting and internalization from the cell surface. These studies were extended to the HIV-1 envelope glycoprotein complex, which also has a tyrosine-based signal that is active in endocytosis. Overexpression of the envelope glycoprotein resulted in missorting of lysosomal proteins to the cell surface, a phenomenon that may explain some of the cytopathic effects of HIV-1 on the host cells.

Proposed Course of Research

The identification of the medium adaptor chains as the receptors for tyrosine-based signals has had a major impact in our ability to study the molecular mechanisms of protein sorting. We now plan to undertake a detailed characterization of the signal-receptor interactions, making use of various binding assays developed over the past year. A major priority will be to establish the structural features of the signals that determine the specificity and avidity of their binding to the receptor molecules. Other factors that may affect their sorting function such as their placement within the cytoplasmic domain and their multiplicity will also be investigated. We also plan to conduct structure-function analyses of the medium chains, for the purpose of identifying the signal-binding domains, sites of interaction with other adaptor chains, regulatory regions, etc. This information on the medium chains will be used to construct dominant mutants that could be used to study the role of these chains in various sorting processes in intact cells. Since μ_1 and μ_2 are members of a growing superfamily of related molecules, we will investigate whether the other members also have a signal recognition function. If this is the case, we will determine whether the medium chain homologs are components of novel adaptor complexes and what their subcellular localizations and functions are. We will also attempt the identification of proteins that bind to the acidic signal in furin using techniques similar to those that led to the identification of the medium adaptor chains as the receptors for tyrosine-based signals.

Publications.

Delahunty M and Bonifacino J. Disorders of intracellular protein trafficking in human disease. *Conn Tissue Res* 1995(In press).

Marks MS, Germain RN, and Bonifacino JS. Transient aggregation of major histocompatibility complex class II chains during assembly in normal spleen cells. *J Biol Chem* 1995;270:10475-81.

Marks MS, Roche P, van Donselaar E, Woodruff L, Peters PJ, and Bonifacino JS. A lysosomal targeting signal in the cytoplasmic tail of the β chain directs HLA-DM to the MHC class II antigen processing compartment. *J Cell Biol* 1995(In press).

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Rajasekaran AK, Humphrey JS, Wagner M, Miesenböck G, Le Bivic A, Bonifacino JS, Rodriguez-Boulan E. TGN38 recycles basolaterally in polarized MDCK cells. *Mol Biol Cell* 1994;5:1093-103.

Voorhees P, Deignan E, van Donselaar E, Humphrey J, Marks MS, Peters PJ and Bonifacino JS. An acidic sequence within the cytoplasmic domain of furin functions as a determinant of TGN localization and internalization from the cell surface. *EMBO J*(In press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01608-5

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Gene Regulation in Response to Environmental Stress

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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C. Essex, Pre-IRTA, CBMB

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A. Zhang, IRTA, CBMB

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LAB/BRANCH

Cell Biology and Metabolism Branch

SECTION

Unit on Environmental Gene Regulation

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:

4

PROFESSIONAL:

4

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This group studies how organisms sense environmental signals and transduce the signals into changes in gene expression and cell physiology. The group has continued their characterization of the two regulators central to the *Escherichia coli* response to oxidative stress: OxyR, a 34 kDa transcriptional activator which is switched on by oxidation, and OxyS, a novel 109 nucleotide RNA regulator which acts as an activator and repressor of gene expression. In addition to studying the mechanisms of OxyR and OxyS action, the group has begun to determine what known regulators are important to the response to hydrogen peroxide in *Saccharomyces cerevisiae*. Using *Arabidopsis thaliana* as a model system, the group is also characterizing mutants defective in their developmental response to blue light.

GENE REGULATION IN RESPONSE TO ENVIRONMENTAL STRESS

Project Description

Objectives:

This project is aimed at understanding the molecular mechanisms whereby organisms perceive an environmental signal and transduce this information into a change in gene expression. In one subproject, we are characterizing the *E. coli* and *S. cerevisiae* responses to oxidative stress, and in a second subproject, we are characterizing *Arabidopsis* mutants to elucidate how plant development is regulated by light.

Methods Employed:

Recombinant DNA techniques employed include the isolation of RNA and DNA, preparation of recombinant plasmids, molecular cloning, nucleic acid electrophoresis, DNA sequencing, Northern and Southern blotting, chemical synthesis of deoxyoligonucleotides, DNA amplification via the polymerase chain reaction, cDNA library construction and subtraction. Biochemical approaches used include fast protein liquid chromatography (FPLC) for the protein purification, DNA binding and *in vitro* transcription assays, and immunoblotting. Standard genetic crosses and the generation of *lacZ* marker gene fusions by transposition or by recombination onto Lambda followed by integration are also being carried out.

Oxidative Stress Responses in Bacteria and Yeast.

Reactive oxygen species (O_2^\bullet , H_2O_2 , and HO^\bullet) can lead to the damage of almost all cell components (DNA, lipid membranes, and proteins) and have been implicated as causative agents in several degenerative diseases. Most organisms have an adaptive response to defend against oxidants. Treatment of both bacterial and yeast cells with low doses of H_2O_2 results in the induction of a distinct group of proteins, the decreased expression of other proteins and resistance to killing by subsequent higher doses of H_2O_2 . The bacterial response to H_2O_2 involves at least two regulators. The expression of nine of the H_2O_2 -inducible proteins is controlled by the OxyR protein which is homologous to the "LysR" family of bacterial regulators. The oxidized but not the reduced form of the OxyR protein activates transcription, suggesting that oxidation of OxyR brings about a conformational change that leads to RNA polymerase activation. Treatment with hydrogen peroxide also leads to the induction of a unique RNA regulator denoted OxyS. Previous studies by this group have shown that OxyS is 109 nucleotides in length and is not translated, and several target genes that are activated or repressed by the OxyS RNA have been identified. Less is known about the regulation of the yeast defenses against hydrogen peroxide, however, regulators of metal homeostasis may also be critical to the oxidative stress response.

Characterization of OxyR domains. We have characterized six different non-binding and nine constitutively-active mutants of OxyR. Five of the mutations causing the DNA-binding defect

map near the N-terminal helix-turn-helix motif conserved among the LysR family members, confirming that this region is a DNA-binding domain in OxyR. The sixth non-binding mutant (E225K) was found to be predominantly dimeric in contrast to the tetrameric wild type protein, suggesting that a C-terminal domain defined by the E225K mutation is involved in multimerization. The mutations causing the constitutive phenotype are located in the carboxy-terminal two thirds of the protein and five of the mutations map near a cysteine residue (C199) critical for the redox-sensitivity of OxyR. *In vivo* as well as *in vitro* transcription experiments showed that the constitutive mutant proteins were able to activate transcription under both oxidizing and reducing conditions, and DNase I footprints showed that the activation is due to the ability of the mutants to induce cooperative binding of RNA polymerase. The mutant proteins are now useful tools for the biochemical characterization of the redox-active center in OxyR.

Regulation by the OxyS RNA. We have also continued our studies of the novel, 109 nucleotide OxyS RNA. We previously found that OxyS acts in *trans* to repress the expression of *fhlA* (encoding an activator of anaerobic genes), *dps* (encoding a non-specific DNA binding protein), *acrH* (encoding a metal resistance/nodulation/cell division-type transporter), *yhiM* (a gene with homology to GABA transporters), and *gadB* (encoding glutamate decarboxylase). OxyS also activates expression of *uhpT* (encoding a hexose phosphate transporter) and *pqqE* (required to synthesize the co-factor PQQ). We have now subcloned regions of the *fhlA*, *dps*, *yhiM*, and *gadB* promoters in order to define the OxyS response elements. Interestingly, while OxyS represses the transcription of *dps*, *yhiM*, and *gadB*, the RNA acts to repress the translation of *fhlA*. Additional studies of the OxyS RNA have shown the the half-life of the RNA is greater than 20 minutes and only the 3' 46 nucleotides are essential for function. Cross-species hybridization also revealed that while the OxyS RNA is present in closely-related *E. coli*, *Salmonella*, and *Shigella*, it is not detected in *Klebsiella*, *Pseudomonas*, or *Enterobacter*.

Roles of Regulators of Metal Homeostasis in the Yeast Response to Oxidative Stress. Relatively little is known about the cellular mechanisms used by yeast to protect against oxidative damage. Since metals contribute to the production of oxygen radicals, we are investigating the effects of known regulators of metal homeostasis on the *S. cerevisiae* response to oxidative stress. We constructed congenic haploid strains carrying single and double knockouts of genes encoding regulators of metal homeostasis (*AFT1*, *MAC1*, *ACE1*) and regulators conferring pleiotropic metal and drug resistance (*YAP1*, *CAD1*). The growth rates and the oxidant sensitivities of these strains are now being determined in a variety of growth assays. This approach has already shown that *YAP1*, *AFT1*, and *MAC1* play a role in protecting the yeast cells against oxidative stress and will provide a foundation for the identification of additional components of the yeast defense response.

Blue Light Regulation in *Arabidopsis*.

The normal development of plants is generally dependent on light. Spectral studies have shown that plants undergo morphological changes in response red/far red, blue, and UV light. For example, dark grown plants have extremely elongated hypocotyls (stems) compared to light-

grown seedlings. At present, the mechanisms by which the light signals are transduced into changes in plant growth and morphology are largely unknown.

Characterization of *hy4* and *hy5* mutants. We took advantage of the difference in hypocotyl length to screen for *Arabidopsis* mutants that have elongated hypocotyls under blue light. These mutants are impaired either in their ability to sense light of this wavelength or in their ability to transduce the light signal into the appropriate developmental response. 24 independent mutants defective in their developmental response to blue light were isolated from a screen of 300,000 mutagenized seeds. The mutants fell into two complementation groups corresponding to the *HY4* and *HY5* genes. Since *HY4* is thought to encode the blue light photoreceptor, 14 *hy4* mutants are now being characterized to determine the nature of the mutations. These studies are being complemented by subtractive screens between wild type, *hy4* and *hy5* cDNA libraries to identify genes that are differentially expressed between the wild type and mutant seedlings.

Characterization of *tt* mutants. One response of *Arabidopsis* seedlings to blue light is elevated production of purple anthocyanin pigments. Since mutants that do not produce the pigments are easily detected, eleven pigment-defective mutants (*tt1-tt10*, *ttg*) have been isolated. In a collaboration with Drs. B. Shirley (Virginia Polytechnic Institute and State University), H. Goodman, W. Kubasek, F. Ausubel (Massachusetts General Hospital) and M. Koornneef (Agricultural University, Wageningen), we have helped to characterize these *tt* mutants to determine the nature of the defects.

Publications:

Kullik I, Toledano MB, Tartaglia LA, Storz G. Mutational analysis of the redox-sensitive transcriptional regulator OxyR: Regions important for oxidation and transcriptional activation. J Bacteriol 1995;177:1275-84.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01609-04

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Localization and Dynamics of Intracellular Organelles

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LAB/BRANCH

Cell Biology and Metabolism Branch

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INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:

5.0

PROFESSIONAL:

3.0

OTHER:

2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

How membrane-bound organelles of eukaryotic cells maintain their identity and subcellular localization amidst an enormous input and outflow of membrane and protein is a central question in cell biology. Studies in this group have focused on this question and have sought to define the cellular and molecular mechanisms which underlie the organization and distribution of eukaryotic organelles. Particular attention has been paid to the Golgi complex which plays a fundamental role in the processing and sorting of protein moving through the secretory pathway. The Golgi complex in higher eukaryotes consists of stacks of flattened cisternae usually localized to the perinuclear region near the microtubular organizing center (MTOC). Recent studies have suggested this organization and positioning of the Golgi are controlled by dynamic processes. Tubulovesicular structures emerging from Golgi elements along microtubules, for example, enable adjacent Golgi stacks to communicate. In addition, reversible dispersal of Golgi elements occurs during microtubule disruption, mitosis and brefeldin A (BFA)-treatment. To further understand these processes and their relationship to the three-dimensional morphology and function of the Golgi we have taken four major approaches. 1) Imaging of the Golgi complex in living cells. Using the vital dye BODIPY-ceramide to label the Golgi complex in living cells we have performed time-lapse imaging to examine the dynamics of the Golgi complex in normal and BFA-treated cells. The important role of membrane tubulation in the normal dynamics and maintenance of Golgi structure within cells is under investigation. 2) Role of microtubules in Golgi localization and traffic. We have found that microtubules are important for both localizing the Golgi complex to the MTOC and for facilitating anterograde (ER-to-Golgi) and retrograde (Golgi-to-ER) membrane traffic into and out of this central region. Our characterization of Golgi dispersal induced by microtubule-disruption has suggested a mechanism involving membrane transport of Golgi components along retrograde and anterograde membrane pathways rather than diffusion or actin/myosin based transport. The role of microtubules in facilitating and directing membrane traffic to and from the Golgi, therefore, is likely to underlie the subcellular location of this organelle. 3) Role of microtubule motors in Golgi traffic. We have found that the microtubule motor protein, kinesin, associates with the anterograde and retrograde pathways leading to and from the Golgi complex. Conditions of microtubule disruption and low temperature treatments, which slow retrograde traffic, result in a large accumulation of kinesin on Golgi membranes. Factors that might regulate kinesin motor activity as this molecule cycles through anterograde and retrograde pathways are under investigation. 4) Role of Golgi positioning in late processing events of the secretory pathway. We have found that microtubule-dependent positioning of the Golgi complex to the MTOC is likely to facilitate the interaction of components moving through secretory and endocytic pathways.

LOCALIZATION AND DYNAMICS OF INTRACELLULAR ORGANELLES

Objectives:

The unit on Organelle Biology at CBMB studies the mechanisms regulating the organization and distribution of organelles and transport intermediates comprising the endomembrane system of higher eukaryotes. Over the past year our group has focused on understanding the dynamics of membrane proteins comprising the Golgi complex, an organelle which plays a central role in the transport, processing and sorting of secretory products leaving the ER. We have pursued three major projects: (1) construction of Golgi protein chimeras containing the *Aequorea victoria* green fluorescence protein (GFP) to examine by fluorescence microscopy the mobility and diffusion rates of Golgi membrane proteins in living cells; (2) development of an assay system to study the extent and role of retrograde (Golgi-to-ER) transport by Golgi membrane proteins; and (3) elucidation of the mechanism(s) underlying Golgi dispersal.

Golgi protein-GFP chimeras

To gain insight into the dynamics and mobility of different proteins comprising Golgi membranes, we constructed Golgi protein chimeras containing GFP and imaged these proteins in living cells using fluorescence microscopy. GFP was attached to (1) the luminal tail of galactosyltransferase, a Golgi resident enzyme (GFP-galtf); (2) the cytoplasmic tail of the KDEL receptor, which resides at steady-state within the Golgi, but recycles to the ER in response to occupancy by KDEL-containing ligands (GFP-KDEL_R); and (3) the cytoplasmic tail of a putative recycling deficient KDEL receptor (GFP-KDEL_{Rm}). In transiently transfected cells all of the GFP-chimeras were localized primarily to the Golgi complex. Overexpression of lysozyme-KDEL within cells caused GFP-KDEL_R, but not GFP-KDEL_{Rm}, to redistribute into the ER, consistent with these two chimeras functioning respectively, as wildtype and defective KDEL receptors. Time-lapse recordings by confocal microscopy following the dynamics of each of the fluorescent chimeras revealed their distribution to include dynamic tubule processes which extended out and retracted, or disconnected from stable perinuclear Golgi elements. Detached, mobile tubule elements containing the GFP chimeras moved away from the Golgi along curvilinear microtubule tracks at rates of 0.6 $\mu\text{m}/\text{sec}$ before changing shape and direction, or fusing with other peripheral structures.

Insight into the potential role of the tubule processes containing the GFP-Golgi protein chimeras came from experiments following their dynamics in cells treated with brefeldin A (BFA), a drug which redistributes all Golgi proteins into the ER. Minutes after adding BFA to cells Golgi tubule elements containing the chimeras became more numerous. These tubules resembled Golgi tubules in untreated cells, moving along microtubule tracks at 0.6 $\mu\text{m}/\text{sec}$ to the cell periphery. Unlike Golgi tubules in untreated cells, the BFA tubules failed to detach from central Golgi structures and within 3-4 min transformed the entire Golgi complex into a tubule network. Soon thereafter, fusion of ER and Golgi membranes occurred resulting in Golgi membranes emptying into the ER within 30 sec. These results raise the possibility that Golgi tubule elements observed in control

cells normally serve as intermediates in Golgi-to-ER retrograde traffic. In untreated cells, such tubule traffic would be highly regulated and involve a mechanism for detachment of retrograde tubules from Golgi cisternae. In BFA-treated cells, such traffic would no longer be regulated, and proliferation of retrograde tubules would result in the inability of the Golgi complex to maintain its distinct identity from the ER. Our ability to image retrograde traffic of Golgi membrane proteins in living cells induced either by BFA or by overexpression of KDEL ligand should prove useful in verifying this proposal.

GFP-Golgi protein FRAP studies

Currently, there is great interest in defining the mechanism(s) underlying the "retention" of Golgi membrane proteins in the Golgi complex. Two alternative models have been suggested: one involving immobilization by self interactions, or kin recognition between different Golgi proteins; and the other by lateral partitioning of Golgi proteins into specific lipid domains. To distinguish between these models we investigated the diffusional mobility of the GFP-chimeras within Golgi membranes using photobleach recovery techniques. Regions of elongated Golgi elements expressing each of the constructs were bleached using a confocal microscope and fluorescence recovery within this region observed by time-lapse fluorescence microscopy. Rapid recovery into the bleached zone was observed for all of the chimeras, indicating lateral diffusion of these proteins across Golgi cisternae. The diffusion rates were quantified using a laser microscope designed for fluorescence photobleaching recovery (FPR) measurements. A stripe of two microns wide was bleached across Golgi membranes. Within this geometry, fluorescence recovered to about 70% of pre-bleach levels. D from the rapid recovery of fluorescence was $1.2 \times 10^{-9} \text{ cm}^2/\text{sec}$ for GFP-KDEL_R, $1.1 \times 10^{-9} \text{ cm}^2/\text{sec}$ for KDEL_{Rm}, and $1.65 \times 10^{-9} \text{ cm}^2/\text{sec}$ for GFP-galtf. These values are approximately that expected for unhindered lateral diffusion of a multispan protein in a membrane and are similar to that of rhodopsin, in vertebrate visual membranes. Our estimates of D were 3-4 fold higher than that previously measured for VSV G protein labeled with a fluorescent antibody fragment while residing within the Golgi. ALF treatment did not affect D , but reduced the recovery of fluorescence to 30-40% of pre-bleach levels. This result is consistent with ALF's effect, which is to fragment Golgi membranes into vesicles smaller in diameter than the bleached line. Rapid mobility of the GFP-Golgi protein chimeras in Golgi membranes suggests that these proteins normally do not interact with components which retard their diffusion.

Significantly, FPR measurements of GFP-galtf redistributed into the ER by BFA, revealed its diffusional mobility to be three times slower than that measured in Golgi membranes. GFP-galtf has a 27 kD ectodomain, while GFP-KDEL_R has essentially none. Since the diffusional mobility of GFP-KDEL_R was the same whether it was in Golgi or in ER membranes (as a result of BFA treatment), it is possible that interactions between the ectodomain of a membrane protein and luminal proteins comprising the ER can alter the diffusional mobility of the membrane protein. Consistent with this possibility, we found that when GFP-KDEL_R was redistributed into the ER by overexpression of KDEL ligand (which behaves as a soluble ER resident protein), the diffusional mobility of KDEL_R in the ER was two fold slower than its mobility either in Golgi membranes or in ER membranes in the absence of overexpressed ligand.

Retrograde transport of Golgi membrane proteins

Proper functioning of the secretory pathway in higher eukaryotes requires a significant retrograde flow of membrane and protein from the Golgi complex back to the endoplasmic reticulum (ER). This ensures return of escaped ER resident proteins, as well as membrane components necessary for continued anterograde traffic. A variety of molecules, including ERGIC-53, p58, and KDEL_R, have been shown to cycle constitutively between the ER and Golgi complex, although the exact routes these molecules travel is unclear. Whether Golgi resident proteins cycle through these pathways, as well, is unknown. To address this question we constructed chimeric Golgi proteins containing the luminal domain of the temperature sensitive viral glycoprotein, tsO45VSV G, which misfolds and is retained within the ER at its restrictive temperature of 40°C, but at 32°C folds correctly and moves through the Golgi complex to the plasma membrane. We reasoned that if Golgi-targeted VSV G chimeras were to cycle between the ER and Golgi complex, then shifting the temperature to 40°C should result in their misfolding and retention in the ER as they cycled through this compartment. In transiently transfected cells at 32°C, VSVG chimeras (including VSV G attached to the transmembrane and cytoplasmic domains of TGN 38, KDEL_R and KDEL_Rm) were found localized to the Golgi complex. Upon shifting the temperature to 40°C, these proteins redistributed into the ER, without altering the distribution of other Golgi membrane markers. The kinetics of this process varied with each chimera, with complete redistribution occurring in as little as fifteen minutes, or as long as three hours. This process was fully reversible, as all chimeras showed a Golgi staining pattern within one hour of shift back to the permissive temperature. These observations suggest that Golgi membrane proteins cycle at some finite rate through pathways connecting the ER and Golgi complex. We are currently using the VSV G-Golgi chimeras to develop an assay system for retrograde transport in order to define the biochemical requirements of this transport pathway.

Mechanism(s) of Golgi dispersal

Despite its distinctive morphology and localization within cells, the Golgi complex is capable of rapid disassembly and reassembly during mitosis, as well as under pharmacologically-induced conditions (e.g. BFA, ililumiquinone or okadaic acid), and reversibly redistributes to numerous peripheral sites in response to microtubule depolymerization. How Golgi fragmentation and dispersal during these processes is accomplished is far from being understood. To begin to understand the mechanisms underlying Golgi dispersal we analyzed this process in cells whose microtubules were depolymerized. It has been known for years that microtubule disruption has dramatic effects on Golgi morphology, with hundreds of functional Golgi islands appearing throughout the cytoplasm. Surprisingly, membrane transport from the endoplasmic reticulum (ER) to the Golgi complex and subsequently to the cell surface is generally not affected. We analyzed this dispersal process using quantitative fluorescence microscopy and digital image processing. Golgi membrane components were found to redistribute to a distinct number of peripheral sites that were not randomly distributed, but corresponded to sites of protein exit from the ER. Whereas Golgi enzymes redistributed gradually over several hours to these peripheral sites, ERGIC-53 (a protein which constitutively cycles between the ER and Golgi) redistributed rapidly (within 15 minutes) to these sites after first moving through the ER. Interestingly,

processing by Golgi enzymes of proteins moving through the secretory pathway was initially blocked after microtubule disruption. Once Golgi components had redistributed to peripheral sites, however, processing was as efficient as in untreated cells. This suggests that Golgi dispersal upon microtubule disruption is required for reestablishing secretory flow from the ER to the Golgi complex. Experiments examining the effects of microtubule disruption on the membrane transport pathways connecting the ER and Golgi suggested a potential role for these pathways in the dispersal process. Anterograde clustering of peripheral pre-Golgi elements into the central Golgi region was blocked by microtubule disruption, whereas retrograde traffic from the Golgi to the ER was only slightly inhibited. Golgi dispersal, therefore, could result from a slow but constitutive flux of Golgi resident proteins through ER/Golgi cycling pathways. In the absence of microtubules, Golgi membrane proteins would accumulate at peripheral ER exit sites due to failure of these structures to cluster into the centrosomal region. Regeneration of Golgi stacks over time in these peripheral sites would re-establish secretory flow from the ER to the Golgi and underlie the Golgi fragmentation process.

Publications:

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01610-03

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Intracellular Metal Metabolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Cell Biology and Metabolism Branch

SECTION

Section on Organelle Receptor Structure and Function

INSTITUTE AND LOCATION

NICHD, Bethesda, MD

TOTAL STAFF YEARS:

5.25

PROFESSIONAL:

5.25

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In order to be incorporated into essential cellular enzymes, iron must first traverse various membrane barriers, and yet the molecular mediators of these transport processes have not been identified. We have utilized the genetically tractable eukaryote, *Saccharomyces cerevisiae*, as a model to identify genes required for the high-affinity transport of iron across the plasma membrane. These genes include CTR1, CCC2, FET3 and FTS3. The FET3 gene encodes an oxidase activity required for high-affinity iron uptake, and the amino acid sequence exhibits similarity to the multi-copper oxidases laccase, ascorbate oxidase and, to some extent, ceruloplasmin. Mutations in CTR1 or CCC2 result in the production of an inactive FET3 apoprotein due to failure to incorporate copper, and the defect in iron uptake in these mutants is a consequence of deficiency of FET3 oxidase activity. Genetic evidence of interaction of FET3 and FTS3 has been obtained: 1) overexpression of both genes is required to augment iron uptake in a wild-type strain, 2) deletion of FTS3 results in loss of iron uptake activity and FET3 oxidase function, and 3) specific mutant alleles of FTS3 abrogate iron uptake without effect on oxidase function. Thus, FET3 may act together with FTS3, which encodes a polytopic membrane protein, to form an iron transporting complex. A genetic approach has also enabled us to identify the iron regulator, AFT1, which mediates the homeostatic control of the iron uptake system. This is accomplished through interaction of the AFT1 protein with cis-acting elements in promoter DNA under conditions of iron deprivation, inducing transcription of the target genes in an iron dependent fashion.

INTRACELLULAR METAL METABOLISM

Objectives

(1) To use the methodology of yeast genetics to identify genes involved in the regulated uptake and utilization of iron, (2) to elucidate the mechanism of the transmembrane transport of iron, and (3) to determine how cellular iron status is "sensed" and how iron-dependent gene regulation ensues from this.

Major Findings

FET3 - Explaining the Copper Dependence of Iron Uptake

Cells that are severely deprived of copper exhibit deficiencies of copper enzymes, for example, copper-zinc superoxide dismutase. Such copper starved cells also exhibited deficient high affinity iron uptake. The physiologic basis for this deficiency was suggested by the observation that FET3, a gene required for high affinity iron uptake, contains sequence motifs that resemble the copper-coordinating amino acids of a multi-copper oxidase. A biochemical assay for the FET3 oxidase was developed in the lab, and using this assay, it was possible to directly demonstrate a copper requirement for the oxidase activity. Thus, the copper requirement for iron uptake stemmed from the need to provide copper to FET3 protein, a copper-containing oxidase required for iron uptake.

The FET3 oxidase assay also provided a means to identify FET3 apoprotein. In cells deprived of copper, FET3 protein is synthesized and is stable, but it has no activity. Activity could be restored to the apoprotein by addition of copper to the cell lysate. As described in the previous Annual Report, we identified two other genes required for high affinity iron uptake. CTR1 was identified by means of the FRE1-HIS3 selection scheme and encodes a copper transport protein localized to the plasma membrane and functioning in copper acquisition from the environment. CCC2 was identified by utilizing degenerate oligonucleotides derived from conserved domains of P-type ATPases to amplify related gene sequences from *S. cerevisiae* genomic DNA. The sequence of CCC2 exhibits strong and global similarity to the family of copper-transporting P-type ATPases, including the human genes implicated in Wilson disease and Menkes disease. Mutation or deletion of CTR1 or CCC2 resulted in failure of adequate copper to reach newly synthesized FET3 protein. Only inactive apoprotein was produced, and consequently, iron uptake was deficient in these mutants.

Additional indirect evidence suggested to us a model in which CTR1 mediates copper transport into the cell, and CCC2 mediates a second copper transport step across an internal cellular

membrane. Both of these transporters must function in series in order for copper loading of FET3 protein to occur. The evidence for this model derives from data indicating that CTR1 mutants were deficient in cellular copper uptake whereas CCC2 mutants were not. Thus, the existence of apo-Fet3p in CCC2 mutants derived from a copper handling defect hoccuring after uptake of copper into the cell. In addition, we noted that the FET3 protein contained N-linked sugars as evidenced by EndoH sensitivity. Analysis of the sequence of the predicted FET3 protein revealed a single transmembrane domain and predicted N-glycosylation sites on the same side of the membrane as the copper coordination sites. Therefore, copper delivery to the FET3 protein must require a membrane crossing step to gain entrance into the secretory pathway, where both sugar addition and copper insertion are likely to occur. Mediation of this step in copper delivery is a likely site of action for the CCC2 P-type ATPase. This function is analogous to the proposed function of the Menkes and Wilson disease gene products. In the latter case, the copper-transporting P-type ATPase functions to deliver copper into the secretory pathway, where it is inserted into ceruloplasmin, a multi-copper oxidase that is secreted into the blood. Interestingly, mutations in the ceruloplasmin gene in humans have recently been linked to disorders of iron homeostasis.

Identification of FTS3, a Candidate Gene for the Iron Transporter

A biological problem that has preoccupied us for some time is the question of how iron is transported across membranes. Although we were able to identify various mutants with deficiency of high affinity iron uptake, the corresponding genes did not include the iron transporter. CTR1 and CCC2 encode copper transporters, and FET3 encodes a membrane-associated oxidase with no resemblance to the family of permeases. A likely candidate for the iron transporter was discovered in the lab by investigating the properties of E30, an unusual mutant identified by means of the FRE1-HIS3 selection. This mutant exhibited recessive phenotypes that included high levels of ferric reductase activity, high ferrous uptake activity (5-10 times normal) and slow growth which could not be rescued by iron addition to the medium. The slow growth of the mutant resulted in genetic instability and the generation of visible papillae on all types of growth media. We isolated clones from these papillae and found that in some cases they exhibited more rapid growth and severely depressed iron uptake. The basis for the depressed iron uptake was analyzed and found to be the result of second site mutations. When these second site mutations were analyzed genetically, we found that several loci were represented: CTR1, FET3, and an unidentified locus. The wild-type copy of the unknown mutated gene was then cloned by complementation. In order to achieve this, we used the chelator, ferrozine, to limit iron in

the growth medium, thereby allowing the wild-type or complemented mutants to grow and inhibiting growth of the strains with defective iron uptake activity. The complementing gene was called FTS3, for Ferrous Transport Suppressor 3, and a marked allele of this gene was reintegrated into the genome. The absence of meiotic recombination between the marked allele and the original mutation demonstrated that we had cloned the correct gene. The complementing activity was contained within an open reading frame with 6 predicted hydrophobic regions consistent with transmembrane domains. A domain was also noted with sequence similarity to a *Bacillus Subtilis* permease of unknown function. However, the 5' region of the homologous bacterial gene contained an excellent palindromic recognition site for the iron regulator Fur. Finally, we observed that the FTS3 transcript was iron regulated in a manner consistent with its presumed role in uptake i.e. the transcript was induced by iron deprivation and repressed by iron replete growth conditions. FTS3, then, appeared to be an excellent candidate gene for the cellular iron transporter.

Genetic Evidence for FTS3/FET3 Association

The phenotypes of FTS3 and FET3 mutants were noted to be similar: each was incapable of growth on ferrozine-containing medium and lacked high affinity iron uptake under all growth conditions. This observation raised the possibility that the FET3 and FTS3 proteins might interact, either directly or indirectly. Moreover, we noted that expression of FET3 from a multi-copy plasmid, which conferred increased oxidase expression, did not result in increased iron uptake activity. Only when FTS3 and FET3 were overexpressed together was iron uptake increased above the wild-type level, perhaps due to a requirement for both genes in mediating iron uptake.

Additional evidence for interaction of FTS3 with FET3 was obtained by examining the effect of mutation of FTS3 on the FET3 oxidase. Complete deletion of the FTS3 gene abrogated oxidase activity, although the amount of FET3 protein synthesized was unaffected. The defect appeared to be due to the failure of copper loading of the oxidase protein, since some degree of restoration of oxidase activity could be achieved in vitro by adding copper to the lysate. Three possible explanations for this finding were considered: 1) FTS3 functions in the delivery of copper to FET3 (like CCC2); 2) FTS3 transiently associates with FET3 in order to deliver it to the correct cellular compartment, where it then acquires copper (chaperone function); or 3) FTS3 forms a stable association with FET3 which is required for the correct localization of both gene products. This association is required for copper loading of the oxidase and for iron transport.

Evidence of a Direct Role of FTS3 in Iron Uptake

We have been able to eliminate the first two possible explanations mentioned above by identifying mutant alleles of FTS3 that interfere with iron uptake, while permitting normal expression, copper loading and full activation of the FET3 oxidase. Truncation of the 3' end of the FTS3 open reading frame by insertion of a stop codon at a unique BstXI site (removing 70 amino acids from the carboxy terminus) blocks iron uptake without affecting oxidase activity. Removal of an additional 54 amino acids by truncating at a unique EcoRV site abolishes oxidase activity and iron uptake. In addition, several site-directed mutants were constructed in glutamic acid residues, singled out because of their occurrence in a conserved motif: REGLE. This motif occurs in slightly altered form in the ferritin light chain sequence: REGAE. Structural data suggests that the E residues in this motif are involved in interaction with iron at the nucleation site in the interior of the ferritin shell. We constructed corresponding mutations in the FTS3 protein and found that they inhibit iron uptake without affecting oxidase function. Thus these mutations provide additional evidence that FTS3 protein interacts directly with iron. In a sense, the FTS3 protein can be considered to be bifunctional, since a portion of the protein is required for copper acquisition (and probably correct targeting) of FET3, while another portion of the protein is required for iron uptake.

To serve as the cellular iron uptake transporter, the FTS3 protein must reside on the cell surface. We sought to demonstrate this directly by placing a myc epitope in frame at the 3' end of the FTS3 open reading frame. The tag insertion did not interfere with the ability of the clone to complement an FTS3 deletion strain, and the protein could be visualized as a bright rim of immunofluorescence at the periphery of the cell, consistent with a plasma membrane localization. We are now actively striving to demonstrate physical association between the FTS3 and FET3 proteins in order to complete the picture of an FTS3/FET3 iron transporting complex.

The mechanism of iron transport suggested by these emerging molecular details involves what at first appears to be a paradox. The FRE1 reductase reduces iron chelates outside the cell, and the FET3 oxidase reoxidizes the iron. However, the many caveats to this statement make it seem less paradoxical. The reduction of iron is not directly coupled to transport; iron reduction at the cell surface, which may be necessary to mobilize ferric iron chelates, occurs in great quantitative excess over the iron transport activity. For example, nanomole quantities of iron are reduced per million cells versus only picomole quantities that are taken up. Subsequently, the reduced iron likely interacts with the FET3/FTS3 complex. The oxidase activity of this complex

appears to be coupled to the movement of iron across the membrane. Although the substrate for the oxidase has not been definitely ascertained, it is possible that the oxidase acts on residues in the transporter protein that then interact with the transported iron. Alternatively, the FET3 oxidase might act directly on the ferrous iron, generating a species of iron that is transport competent. Work on ferritin has shown that it possesses a ferroxidase activity which generates ferric monomers that can then be transported from one ferritin polymer to another. The transported iron species, ferric monomer, is only transiently formed upon reoxidation of ferrous iron in proximity to a protein surface. Analogously, the presence of the transport protein surface (FET3 protein) in proximity with the oxidase (FET3 protein) could be necessary to directly interact with the ferric iron species that is formed, perhaps through critical carboxylate residues. This process must compete with the process of ferric iron hydration and precipitation that occurs in aqueous solutions.

Cellular Iron Homeostasis - Role and Mechanism of Action of AFT1

Iron is required for incorporation into essential metalloproteins utilized for electron transfer, redox chemistry and other cellular functions, but excess iron is toxic. Homeostatic regulation of the cellular iron status, therefore, is important to cell survival. In yeast, this objective is achieved through the transcriptional regulation of essential components of the uptake system. We have learned that this regulation affects not just one rate limiting component but all the components of the iron uptake system thus far identified. In previous Annual Reports, we noted iron regulation of FRE1 and FRE2, the genes encoding the surface reductase. Also, FET3, the copper dependent oxidase was found to be iron regulated. This year, we have observed similar coordinate, iron dependent regulation of CCC2, the copper transporting P-type ATPase and FTS3, the putative iron transporter. Two additional iron regulated genes were identified by means of a genetic screen originally devised by M. Snyder. In this scheme, yeast genomic DNA was marked with random lacZ insertions by means of Tn3 "hops" and then reintegrated into the genome of a diploid yeast strain. These lacZ insertion mutants were screened for regulated lacZ activity on iron rich versus iron poor plates. The two genes identified by this approach include one with strong homology to the MDR family of ATP-dependent pumps (distinct from the P-type ATPase family). Determination of the localization and function of the gene product has not yet been achieved, but conceivably it could serve an iron pumping function across an internal cellular membrane.

For each of the iron regulated genes identified, the regulation occurs at the level of transcription and is mediated by AFT1, the iron regulator gene which we identified and cloned last year (see

previous Annual Report). The identification of the dominant AFT1-lup allele, in which the expression of iron regulated genes is constitutive, and the construction of an interrupted (loss-of-function) allele, in which expression of these genes is severely impaired, led us to believe that AFT1 must be an activator of transcription. This led to the prediction that, under under starvation conditions, AFT1 should interact either directly or indirectly with the promoter regions of iron regulated genes. By analysis of gene fusions, the cis-acting element mediating AFT1 regulation has been defined, and direct interaction of the AFT1 protein with this element has been demonstrated utilizing a gel mobility shift assay. In addition, by means of an in vivo footprinting assay, the regulated interaction of AFT1 with its target element on the yeast genome has been demonstrated. In view of this data, it is likely that AFT1 mediates iron regulation of transcription through direct interaction with the promoter DNA of its target genes.

The sensing of cellular iron levels may also be mediated through AFT1. The AFT1-lup allele contains a mutation that alters the cysteine to a phenylalanine at position 291 of the protein. Perhaps this critical cysteine residue together with other cysteines in the AFT1 protein coordinates iron when the cell is iron replete, stabilizing an AFT1 protein conformation which is incapable of interacting with DNA. In a situation in which iron is limited, perhaps the AFT1 protein switches conformation and binds to DNA, thereby initiating transcription. Defining the mechanism of such a transcriptional regulatory switch will require further genetic and biochemical work.

The toxicity of iron is thought to be mediated by the participation of iron in free radical generation in the presence of oxygen. Free radicals are thought to damage vital cellular macromolecules. However, other mechanisms of toxicity, such as misincorporation of iron in zinc proteins, could also be important. Moreover, the specific cellular targets of iron overload toxicity are not known in any detail, nor are the cellular defense mechanisms well characterized. To address some of these questions genetically we have initiated a screen for mutants which are synthetically lethal with the AFT1-lup allele, expressed under the control of the Gal10 promoter. Mutants have been identified and are being characterized. In addition, candidate genes such as the RAD genes that might protect against iron toxicity are being evaluated for the synthetic lethal effects when combined with the AFT1-lup allele.

Relevance to Human Physiology and Disease

The genetic analysis of the iron uptake system in yeast has continued to yield insights that may be relevant to human physiology and disease. This relevance can be viewed on two

levels. On one level, the yeast genes are homologous to human genes to varying degrees. FRE1, the structural gene for the yeast iron reductase is homologous to gp91-phox, the structural gene for the human granulocyte respiratory burst oxidase. The visible spectra of these two membrane cytochromes are virtually superimposable. As part of a collaboration with Anthony Segal, we have successfully overexpressed the yeast protein under the ADH1 promoter and placed an epitope tag that allows us to analyze protein expression. He will determine the midpoint potential of the yeast protein by titrating the FRE1 heme spectrum. This may shed light on the mechanism of action and substrate specificity of the reductase. Domain swapping with the human protein may yield structure-function information regarding such issues as heme coordination and subunit interaction, information which has been difficult to ascertain for the human multi-subunit complex. Other areas of relevance to human disease stem from the CCC2 homology to the human Menkes and Wilson disease genes. Areas that could be pursued relate to the analysis of human disease mutations in the yeast system (corresponding mutations could be made in the yeast genes). Localization of the yeast gene product might have implications for the mechanism and the cellular site of action of the Menkes and Wilson disease gene products.

On a more general level, the linkage of cellular copper and iron homeostasis observed in yeast is apparently conserved in humans. Copper deficiency, mutation in the Wilson disease gene or mutation in the ceruloplasmin gene can lead to severe perturbations in iron physiology. The description of kindreds with ceruloplasmin mutations and abnormal iron handling has only recently been reported. Finally, if FTS3 is the iron transporter of yeast, this raises the question of whether an FTS3 homologue exists in humans. If ceruloplasmin is the human FET3 homologue and the mechanism of iron transport requires interaction with a multicopper oxidase, then how might this ceruloplasmin interact with a human iron transporter? Identification of the human iron transporter would be of great interest because of the relevance to diseases of iron metabolism, such as hemochromatosis. This is a common genetic disease resulting from the failure of the homeostatic regulation of iron uptake from the gut. The genetic locus has been known to reside in the HLA region of Chromosome 6 but the gene has still not been cloned, due to the lack of recombinants in this region. More generally, the human iron transporter gene would be of importance because of its effects on general processes that require iron i.e. cell proliferation, respiration, metabolism and defense.

Publications

Anderson GJ, Dancis A, Roman DG, and Klausner RD. Ferric iron reduction and iron uptake in eucaryotes: studies with the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *Adv Exp Med Biol* 1994;356:81-89.

Dancis A, Haile D, Yuan DS, and Klausner RD. The *Saccharomyces cerevisiae* copper transport protein (Ctrlp): Biochemical characterization, regulation by copper and physiologic role in copper uptake. *J Biol Chem* 1994;41:25660-25667.

Klausner R and Dancis A. A genetic approach to elucidating eukaryotic iron metabolism. *FEBS Letters* 1994;355:109-113.

Yamaguchi-Iwai Y, Dancis A, and Klausner RD. AFT1: A mediator of iron regulated transcriptional control in *Saccharomyces cerevisiae*. *EMBO J* 1995;14:1231-1239.

Yuan DS, Stearman R, Dancis A, Dunn T, Beeler T, and Klausner RD. The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proc Natl Acad Sci USA* 1995; 92:2632-2636.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01-HD-01611-01

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The VHL Tumor Suppressor Gene

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Richard D. Klausner, M.D., Chief, CBMB, NICHD
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Oklahoma Medical Research Foundation (Drs. Ronald and Joan Conaway)

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Cell Biology and Metabolism Branch

SECTION

Section on Organelle Receptor Structure and Function

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3.25

PROFESSIONAL:

2.25

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The VHL gene was first identified in 1993 as the gene responsible for a rare inherited cancer syndrome called Von Hippel-Lindau disease. Inheritance of a single defective VHL gene results in the predisposition to the development of a variety of cancers including clear cell renal carcinoma, pheochromocytoma, and angioblastomas of the central nervous system, among others. Subsequently, it was shown that the majority of tumors derived from patients with sporadic clear cell renal carcinoma also contained both loss of heterozygosity of the VHL locus and failure of expression and/or mutations in the remaining VHL allele. The goal of this project is to understand the biochemistry and cell biology of the VHL gene product and thereby to elucidate its role in cell transformation. Studies over the past year include the completion of the cloning and sequencing of the human and rat VHL gene, the biochemical characterization of the gene product and its intracellular localization. The identification and cloning of gene products that VHL interacts with has illuminated an unexpected new area of gene control--the regulation of transcriptional elongation.

THE VHL TUMOR SUPPRESSOR GENE

Findings

Cloning and Comparison of the Rat and Human VHL cDNAs

Six independent rat cDNA clones were obtained by hybridization with human VHL cDNA using sequences derived from EXONS 1 and 2. The longest rat cDNA of approximately 2.81 Kb hybridized to a single mRNA species from rat tissues and cell lines of 2.91 Kb in length. In contrast to the human protein, the presence of upstream in-frame termination codons allowed us to uniquely assign a start codon for the rat gene. The fact that amino acid nucleotide homology between the rat and human gene begins with the rat start codon allowed us to finally predict the assignment of the human start codon. The rat VHL cDNA predicts a protein of 185 amino acids compared to the human protein of 213 amino acids. The size difference between the two proteins results largely from a single region of disparity near the amino terminus. In the human protein a unique gly-x-glu-glu-x acidic motif is repeated eight times, while this sequence occurs only once in the rat protein. Aside from the acidic repeats, the predicted rat VHL protein shares 88% sequence identity with the corresponding sequence in the predicted human VHL protein.

Subcellular Localization of VHL

Epitope tagged human and rat proteins were introduced into several cell lines and examined by immunofluorescence microscopy. The surprise finding was that in any population of transiently transfected cells, a variety of patterns were observed. Many cells that are predominantly or uniquely nuclear localization while other cells had a cytosolic localization with sparing of the nucleus. Finally, a population of cells appear to have localization in both nucleus and cytosol. This varied distribution of the protein was confirmed by subcellular fractionation.

Formation of Protein Complexes

When VHL was either transiently or stably expressed in a variety of recipient cell types it was observed to associate with numerous endogenous proteins. This was assessed by steady state metabolic labeling followed by immunoprecipitation using antibodies against the epitope tag introduced into either the amino or carboxy termini of either the rat or human VHL cDNAs. Numerous proteins were thus observed specifically assembling with VHL. These included a pentamer of proteins between 55 and 60 kD and two proteins of approximately 9 and 16 kD. Sucrose gradient centrifugations separated these into several complexes including one that appeared to contain stoichiometric amounts of VHL and the 9 and 16 kD proteins. When a variety of naturally occurring point mutations, observed either in tumors from sporadic kidney cancer or from the germ line of VHL patients were examined, they demonstrated a complete or partial loss of the ability of VHL protein to specifically assemble with the 9 and 16 kD partners.

Purification and Identification of VHL Associated Proteins

The specific loss of the 9 and 16 kD proteins with presumably inactivating VHL mutations led us to purify these two proteins. This was accomplished by the creation of stable transfectants of epitope tagged VHL followed by immunoprecipitation after large scale cell culturing. Immunoaffinity purification followed by SDS page electrophoresis resulted in the purification of sufficient amounts of the 9 and 16 kD proteins to allow peptide sequencing. Each of these proteins were digested with a protease (lysC) and the peptides eluted and separated by HPLC for sequential Edman degradation.

The protein sequences were thus determined. The 16 kD protein proved to be identical to a protein in the database which is a subunit of an RNA polymerase (polII) elongation factor referred to as SIII or elongin. Elongin is a heterotrimer consisting of three proteins: elongin A (110 kD), elongin B (18 kD), and elongin C (14 kD). The 16 kD VHL associated protein is identical to elongin B. Sequencing of the 9 kD protein demonstrated that it was identical to the unpublished sequence (received from Ron and Joan Conaway) of elongin C. We used antibodies specific for elongin B and C to demonstrate that indeed the VHL associated proteins were these two subunits of the elongation complex.

Assembly of VHL with Elongin Subunits

We used a variety of techniques to establish the ability of VHL protein to assemble *in vitro* with elongin subunits. These included the use of purified bacterially produced recombinant proteins or the use of *in vitro* transcription/translation coupled reactions. For these studies, we utilized and examined all possible interactions between VHL and elongins A, B, and C. These studies demonstrated that VHL was not capable of assembling with elongin A, regardless of the presence of other elongin subunits. There is some binding of VHL to elongin C in the absence of any other subunit, while there was no detectable interaction of elongin B with VHL. When elongin B and C were both present, a large amount of assembly with VHL was seen with the apparent production of a trimer of stoichiometry 1:1:1. This was the same pattern of assembly that was seen between elongin A and the B and C subunits. Elongin B and C share no sequence similarities with VHL, but VHL does contain a 13 amino acid stretch that is very similar (10 out of 13 identicals) to a sequence found in elongin A. A peptide containing this sequence derived from VHL is capable of competing for the assembly of either elongin A or VHL with elongin B and C.

Effect of VHL on Elongin-Stimulated Transcriptional Elongation

To investigate whether VHL affects elongin activity, we included VHL in two different assays of transcriptional elongation: (1) the adenovirus 2 major late promotor runoff transcription assay and (2) the oligo(dC)-tailed template assay which permits direct measurement of elongin activity in the absence of general initiation factors. When VHL was titrated into such reactions in the presence of the three elongin subunits there was a dose-dependent complete inhibition of polII transcription elongation activity. This inhibition could be quenched by the titration of elongin B and C subunits.

All of these data demonstrate that VHL can compete with elongin A for assembly with elongin BC in such a way VHL appears to be capable of regulating transcription at the level of polII elongation.

Publications

Duan DR, Humphrey JS, Chen DYT, Weng Y, Sukegawa J, Lee S, Gnarra J, Linehan WM, and Klausner RD. Characterization of the VHL tumor suppressor gene product: Localization, complex, formation, and the effect of natural inactivating mutations. PNAS 1995;92:6459-6463.

Duan DR, Pause A, Burgess WA, Aso T, Chen DYT, Garrett KP, Conaway RC, Conaway JW, Linehan WM, and Klausner RD. Inhibition of transcription elongation by the VHL tumor suppressor protein. Science 1995;269:1402-1406.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00610-15 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Growth, Puberty, Their Disorders: Physiology, Pathophysiology, and Molecular Biology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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 Others: (see attached)

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SECTION

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TOTAL STAFF YEARS:

7.4

PROFESSIONAL:

6.1

OTHER:

1.3

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective of this project is to advance understanding of the mechanisms that underlie normal and abnormal puberty, and to apply this knowledge to improve existing therapy for disorders of puberty. Since somatic growth is a major determinant of the timing of pubertal onset, a further objective is to clarify the mechanisms of normal growth and of growth failure. Principle areas of laboratory investigation include the mechanisms of normal growth and puberty. Principle areas of clinical investigation include the mechanism of premature thelarche and of the gonadotropin-independent forms of precocious puberty, the developmental changes in hypothalamic regulation of gonadotropin secretion, the behavioral changes associated with normal and abnormal pubertal development, the mechanisms of prepubertal and pubertal growth, the role of pubertal sex steroids in the acquisition of normal adult bone density, the treatment of central precocious puberty with an analog of luteinizing hormone-releasing hormone (LHRH), the treatment of familial male isosexual precocious puberty with combined antiandrogen and aromatase inhibitor, the evaluation of new approaches to the diagnosis of growth hormone deficiency and to the differential diagnosis of delayed puberty, the treatment with synthetic parathyroid hormone of children with hypoparathyroidism, the treatment with growth hormone of children with Turner syndrome and with non-growth hormone-deficient short stature, and the treatment with insulin-like growth factor-1 of children with growth hormone insensitivity and of children with non-growth hormone-deficient short stature.

The principal areas of laboratory investigation include the structure and function of the regulatory sequences of the human gonadotropin-releasing hormone (GnRH) gene, molecular analysis of the gene for the luteinizing hormone receptor in familial male precocious puberty and in Leydig cell hypoplasia, and of the gene for the calcium-sensing receptor in sporadic and autosomal dominant hypoparathyroidism, and hormonal regulation of epiphyseal transforming growth factor- β , fibroblast growth factor, platelet-derived growth factor, and the growth hormone receptor. To examine the effects of these growth factors in vivo we are studying rabbits bearing small needles implanted into the proximal tibial epiphyses and connected to osmotic minipumps.

Others:

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J. Baron	Senior Clinical Associate	SDE, DEB, NICHD
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P. Feuillan	Special Volunteer	SDE, DEB, NICHD
N. Gerstle	Special Volunteer	SDE, DEB, NICHD
P. Gore	Summer IRTA	SDE, DEB, NICHD
B. Hossein-Zadeh	Summer IRTA	SDE, DEB, NICHD
C. Heinrichs	Special Volunteer	SDE, DEB, NICHD
L. Laue	Adjunct Scientist(IPA-G'town)	SDE, DEB, NICHD
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J. Levine Ross	Special Volunteer	SDE, DEB, NICHD
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Project Description

Objectives:

We seek to increase understanding of normal and abnormal growth and pubertal development, and to apply this knowledge to improve treatment for disorders of growth and puberty. The specific objectives are:

- 1) to determine gonadotropin pulse frequency and amplitude during normal puberty, and to compare the normal pattern with the pattern in children with central precocious puberty, particularly children with a hypothalamic hamartoma;
- 2) to identify the molecular mechanism of testicular autonomy in boys with familial male precocious precocity (FMPP), and of Leydig cell failure in Leydig cell hypoplasia, through analysis of the gene for the luteinizing hormone (LH) receptor;
- 3) to explore the mechanism of premature thelarche;
- 4) to develop improved methods for the diagnosis of growth hormone deficiency and for the differential diagnosis of delayed puberty;
- 5) to determine the efficacy and safety of treating the gonadotropin-independent ovarian function of McCune-Albright syndrome with aromatase inhibitors;
- 6) to determine the efficacy and safety of treating familial male precocious puberty with combined antiandrogen and aromatase inhibitor;
- 7) to determine the efficacy and safety of LHRH analog therapy of central precocious puberty through long-term follow-up of children under treatment;
- 8) to understand the mechanism of the pubertal growth spurt and its relationship to prepubertal growth, to optimize medically-induced prepubertal and pubertal growth in Turner syndrome through clinical trials of growth hormone (with and without low-dose estrogen treatment), to understand the mechanisms of non-growth hormone (GH)- deficient short stature and to improve the long-term outcome, and to assess the efficacy and safety of LHRH agonist-induced pubertal delay, of growth hormone treatment, and of treatment with insulin-like growth factor-1 on adult height in children with non-GH-deficient short stature;
- 9) to improve understanding of the role of estrogen in growth and development during childhood, early puberty, and the pubertal growth spurt by applying a newly developed ultrasensitive recombinant cell bioassay for estradiol;
- 10) to examine the hormonal regulation in epiphyseal cartilage of transforming growth factor- β , fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, and GH receptor;
- 11) to understand the regulation of epiphyseal growth in vivo through the response to added growth factors and to blockade of growth factor action, using both classical physiologic approaches and the creation of transgenic animals;
- 12) to explore the role of pubertal sex steroid secretion in the acquisition of normal adult bone density;
- 13) to study the structure and regulation of the normal human LHRH gene and to compare the observations with the results in genetic disorders causing early or late puberty; to study the regulation of the LHRH gene and of LHRH secretion in a cultured LHRH neuronal cell line;

14) to correct the absence of parathyroid hormone action on bone and kidney in patients with hypoparathyroidism by administration of synthetic parathyroid hormone;

15) to identify the molecular mechanism of autosomal dominant and sporadic hypoparathyroidism through analysis of the gene for the calcium-sensing receptor.

Methods Employed:

- 1) Hypothalamic-pituitary maturation during puberty-steroid and peptide hormone radioimmunoassay and bioassay.
- 2) Mechanism of familial male precocious puberty and of Leydig cell hypoplasia - molecular studies of the LH receptor gene by PCR, sequencing, site-directed mutagenesis, and expression in cultured cells.
- 3) Treatment of precocious puberty - clinical, radiographic, behavioral, and hormonal evaluation.
- 4) Mechanism of normal prepubertal and pubertal growth, and of idiopathic growth failure - bioassay and radioimmunoassay of growth factors; immunostaining of peptide growth factors in epiphyseal cartilage; in situ hybridization, solution hybridization, and/or Northern analysis to quantitate growth factor mRNAs in epiphyseal cartilage; infusion of growth factors or growth factor antagonists into the rabbit proximal tibial epiphyseal growth plate; creation of transgenic animals from embryonic stem cells with heterozygous or homozygous cartilage-specific deletion of growth factors; molecular studies of the growth hormone (GH) receptor (GHR) gene.
- 5) Treatment of growth failure - randomized, double-blind, placebo-controlled trials with adult height as the principal growth endpoint; short-term studies to define the conditions that appear optimal for long-term evaluation.
- 6) Mechanism of autosomal dominant or sporadic hypoparathyroidism - molecular studies of the calcium-sensing receptor gene by PCR, sequencing, site-directed mutagenesis, and expression in cultured cells.

Progress:

1. Mechanism and treatment of precocious puberty

a. Familial male precocious puberty (FMPP) and Leydig cell hypoplasia - molecular mechanism

To determine the structural requirements for activating mutations of the LH receptor (LHR), the mutations were identified from more than 40 families with familial male precocious puberty (FMPP) from widely separated geographic regions. A total of 8 different mutations were identified, which comprise 2/3 of the 12 known activating mutations of the LHR. Eleven of the 12 activating mutations are in a 40-amino acid region from position 542 to 581, which spans the fifth and sixth transmembrane domains and the intervening third intracellular loop. This hot spot for activating mutations identifies a region that plays a critical role in constraining the unliganded receptor in an inactive conformation. Thus, the studies of this rare form of precocious puberty have pinpointed a region of the LH receptor that is critical for signal transduction and hence for the hormonal regulation of human reproduction.

Leydig cell hypoplasia is an autosomal recessive form of male pseudohermaphroditism. To test the hypothesis that this disorder results from inactivating mutations of the LH receptor, we performed studies similar to those described above for FMPP. To date several mutations have been identified that appear to explain this disorder: (1) a nonsense mutation (A1635C) in the fifth transmembrane domain that produces a truncated receptor, with reduced number and affinity of receptors in transfected cells, and a complete lack of signal transduction when exposed to hCG; (2) a missense mutation (Ser616Tyr) that does not alter receptor affinity but markedly impairs hCG-induced cAMP production per binding site. Interestingly, the known inactivating mutations are in the same region, transmembrane domains 5 and 6, as the activating mutations of FMPP, and they appear to interfere primarily with signal transduction. We hypothesize that as larger numbers of these patients are studied there will also be inactivating mutations that interfere primarily with hormone binding.

b. Familial male precocious puberty-treatment with combined antiandrogen and aromatase inhibitor

A long-term pilot study is ongoing to test the hypothesis that the growth rate, bone maturation, and adult height of these boys can be normalized with the regimen of antiandrogen (spironolactone) aromatase inhibitor (testolactone), and LHRH analog (deslorelin [for those boys who develop secondary LHRH-dependent precocious puberty]). We have also initiated a new project to improve this regimen through the use of a more potent aromatase inhibitor (CGS 16949A [fadrozole]).

c. McCune-Albright syndrome - treatment with the aromatase inhibitor testolactone

A long-term pilot study is ongoing to test the hypothesis that the aromatase inhibitor, testolactone, can improve the growth rate, bone maturation, and adult height of girls with this disorder. We have also initiated a new project to improve this regimen through the use of a more potent aromatase inhibitor (CGS16949A [fadrozole]).

d. LHRH analog therapy of central precocious puberty

Analysis of mean adult height (-1.1 SD) in the first 44 children to attain adult height or near-adult height (within 2 cm) indicates a highly significant improvement over pretreatment predicted height (-2.0 SD) but a clear shortfall compared to expected height based upon parental height (+ 0.1 SD). However, the first children treated had the greatest delay of treatment from onset of symptoms (> 3 years), and thus it would be premature to conclude that this is the best result that can be achieved. Continued follow up of those children who were treated later and are still growing will test our hypothesis that there will be an even greater improvement in the final height of these children.

2. Mechanism and optimization of prepubertal and pubertal growth

a. The role of estradiol in prepubertal growth

To test the hypothesis that girls secrete more estrogen than do boys during the prepubertal years, we developed an ultrasensitive recombinant cell bioassay for estradiol, based upon yeast cells genetically engineered for extreme sensitivity to estrogen, with a sensitivity of 0.02 pg/mL, approximately 100-fold more sensitive than previous radioimmunoassays. With this assay, Karen Oerter Klein and Jeffrey Baron showed that estradiol levels in prepubertal girls (0.6 ± 0.6 pg/mL) are significantly higher than the level in boys (0.08 ± 0.2 pg/mL). This may explain the more rapid bone maturation, the earlier entry into puberty, and the earlier cessation of growth of girls compared to boys.

Recently, we have also used this assay to evaluate estradiol suppression by a new aromatase inhibitor (letrozole) in postmenopausal women with breast cancer. Letrozole, at the dose of 100 ug, reduced estradiol from 1.95 to 0.07 pg/mL, suggesting that this drug should prove useful in the treatment of estrogen-dependent conditions. From a broader perspective, this study suggests the potential range of applications of this new assay method. Indeed, in an editorial in the Journal of Clinical Investigation accompanying this publication, Dr. Jean Wilson stated that it should lead to a "renaissance in estrogen physiology."

b. Saltatory versus continuous model of linear growth

Traditionally, linear growth has been considered a continuous process, the cumulative result of millions of unsynchronized chondrocyte divisions throughout the growth plates in the long bones and spine. Recently, this view was challenged in a study that concluded that human growth occurs by a process of saltation (sudden spurts) separated by long periods of no growth. These observations, if correct, would require fundamental revisions in our understanding of growth, since none of the known endocrine regulations of growth fluctuate in the manner that would be required to synchronize cell divisions throughout the organism.

We attempted to confirm the hypothesis of saltatory growth by performing 4 different growth measures daily for 30 days in 5 infants. A method of blinding was developed to prevent observer bias. By 4 different statistical

approaches, the observed data agreed significantly better with a saltatory than with a continuous model. Thus, our data did not support the proposed saltatory model and suggested instead that growth occurs continuously.

c. Treatment of Turner syndrome

Based upon the results of our earlier short-term studies, a long-term, randomized, double-blind, placebo-controlled clinical trial has been initiated to assess the effect on adult height of estrogen, growth hormone, and combined estrogen and growth hormone treatment. The project has been organized as a two-site project, with 1/3 of the patients to be followed at Thomas Jefferson University and 2/3 at the NIH, under a joint venture agreement with Eli Lilly and Company, which will provide biosynthetic human growth hormone and certain additional support for the study. Approximately 140 patients out of a projected total of 160 patients have been enrolled in the study.

Although the major endpoints of this study remain blinded until the study conclusion, analysis of certain baseline and interim data has revealed several new findings. First, analysis of the baseline lipid levels from 137 Turner girls and 70 normal girls showed that Turner girls above age 11 have significantly greater cholesterol levels (190 ± 23 vs. 165 ± 26 mg/dL) after adjustment for age, karyotype, and body mass index ($p < 0.01$). These increased levels precede estrogen or growth hormone treatment. Second, questionnaires from both the parents and the girls with Turner syndrome revealed weaker social relationships, school performance, and self-esteem compared to control girls matched for age, socioeconomic status, and verbal IQ. Third, cognitive tests of the Turner girls has revealed a characteristic profile of impaired performance on tests of spatial recall, recognition, and reasoning (representing in magnitude about 1 SD unit relative to the control population). This cognitive profile appeared quite consistent from childhood through adolescence. Future analyses will determine the effect of sex steroid administration on this cognitive phenotype, and will examine the cognitive phenotype in girls with unusual karyotypes involving partial deletion of one X-chromosome in order to determine the relationship between the specific X-chromosomal region deleted and the cognitive phenotype. Thus, the early findings from this trial have revealed new metabolic, behavioral, and cognitive features of the Turner syndrome.

d. The role of growth factors in epiphyseal growth

An extensive body of knowledge suggests that cell division requires stimulation by growth factors that may be endocrine, paracrine, or autocrine in origin. However, the nature, origin, and regulation of the growth factors that control cell division in human epiphyseal cartilage remain unknown. The production by recombinant DNA methods of factors important for the growth of cultured cells has made it possible to begin to assess the clinical and physiological significance of these growth factors. To examine the potential roles of transforming growth factor- β (TGF- β), fibroblast growth factor (FGF), and platelet-derived growth factor in epiphyseal cartilage, we have initiated a collaborative project to examine the hormonal regulation of these peptides by immunostaining with specific antisera. We will also attempt to examine hormonal regulation of the mRNAs for these peptides in epiphyseal cartilage by in situ hybridization and/or solution hybridization. Lastly, we have developed a method to infuse these factors, or their antagonists, into the rabbit epiphyseal growth plate, and to measure the subsequent growth response.

Infusion of basic FGF into the rabbit proximal tibial epiphyseal growth plate caused a dramatic induction of growth plate cartilage ossification. This observation suggests an important role for basic FGF in the biology of longitudinal bone growth, particularly in the terminal differentiation of hypertrophic chondrocytes and their invasion and replacement by bone.

e. Mechanism of glucocorticoid-induced growth failure and subsequent catch-up growth

Supraphysiologic levels of glucocorticoid cause a profound arrest of epiphyseal growth. This poses a problem both in endogenous Cushing's syndrome in children and in children who require supraphysiologic glucocorticoid doses as treatment for asthma, juvenile rheumatoid arthritis, and other disorders.

Previous studies in our laboratory showed that glucocorticoid-induced growth suppression could be induced in a single proximal tibial epiphysis by the local infusion of dexamethasone. The mechanism of this local growth

arrest did not involve suppression of mRNA for growth hormone receptor in the affected epiphysis. Recently, we have used this growth suppression model to examine the hypothesis that catch-up growth after glucocorticoid withdrawal results from a neuroendocrine mechanism. Contrary to this hypothesis, catch-up growth after termination of the dexamethasone infusion was observed only in the epiphysis that had received dexamethasone. No change in growth rate was observed in the control contralateral epiphysis, or in the ipsilateral distal tibial epiphysis. Thus, catch-up growth following glucocorticoid withdrawal was a local phenomenon that occurred only within the affected growth plate.

f. Mechanism of non-GH-deficient short stature

We previously published, in the New England Journal of Medicine, a comparison of diagnostic methods for growth hormone (GH) deficiency in 54 short children. The study found low sensitivity and diagnostic accuracy of the mean spontaneous 24-hour GH level compared to the peak GH level during 3 GH stimulation tests.

This original study involved only prepubertal children. More recently, we have extended our studies of spontaneous GH secretion (now measured as the mean 12-hour nighttime GH level) to include a large cohort of short children in each of the 5 stages of puberty. As in the original study of prepubertal short children, we observed a very low incidence of abnormally low mean nighttime GH levels among pubertal children with idiopathic short stature. Thus, low spontaneous GH secretion is a highly unusual cause of growth failure in pubertal, as in prepubertal, short children, and the routine use of spontaneous GH measurement to detect GH neurosecretory dysfunction in such children is unwarranted.

To test the hypothesis that the diagnostic usefulness of spontaneous growth hormone (GH) measurements could be improved by conducting measurements under circumstances that augment GH secretion, we propose to conduct these measurements during fasting and after the brief administration of sex steroid. We hypothesize that these measures will augment spontaneous GH levels in non-GH-deficient short stature but not in true GH deficiency, thus giving rise to a clinically useful test for patients in whom the GH stimulation tests yield equivocal results.

g. Molecular basis of Laron syndrome

Previous studies from several laboratories have revealed a number of different mutations that impair hormone binding or signal transduction by the GH receptor. Recently, we analyzed the GH receptor (GHR) from a geographically isolated Chilean boy with Laron syndrome who was the product of a consanguineous marriage. Sequencing of the GHR revealed a novel 2-base pair microdeletion leading to a premature stop codon within the extracellular domain, upstream of the critical transmembrane and intracellular domains. This unusual mutation thus expands the known molecular mechanisms that underlie this syndrome of extreme growth failure. This observation is part of an ongoing effort to understand the molecular basis for unexplained growth failure in children.

h. Treatment of non-GH-deficient short stature

1) Growth hormone

Recent studies from several centers have shown that biosynthetic human growth hormone (hGH) can increase short-term growth rate in non-GH-deficient short stature. However, short-term effects are not always sustained, and treatment-induced acceleration of pubertal onset could negate short-term gains. Moreover, the increasing off-label use of hGH for this purpose has given an important public health significance to the determination of whether such use is effective and safe. Thus, to address the long-term outcome of such treatment, a randomized, double-blind, placebo-controlled trial has been designed to determine the effect of biosynthetic human growth hormone on the adult height of children with non-GH-deficient short stature. A joint venture agreement with Eli Lilly and Company has been established to provide growth hormone and additional support for the study. Approximately 56 of the total of 80 subjects have been enrolled in this study.

Although we remain blinded to the major endpoints of the trial, we have been permitted to examine the hypothesis that GH treatment induces hypothyroidism in some children with non-GH-deficient short stature. Based upon analysis of the first year's data in 20 subjects, we concluded that GH treatment for 12 months does not produce sustained alterations in thyroid function in non-GH-deficient children.

2) LHRH analog-induced pubertal delay

The hypothesis that adult height can be increased by prolonging the growth period through delay of puberty is supported by our observation of greater adult stature in patients with isolated hypogonadotropic hypogonadism (IHH) compared to normal subjects. To determine whether similar gains can be achieved through pharmacologic delay of puberty by LHRH analog, a randomized, placebo-controlled trial was initiated that is now fully enrolled (50 subjects). The results of this trial will not be known until the subjects have attained adult height.

3) Insulin-like growth factor-I

Recombinant human IGF-I, the growth factor believed to mediate the growth-promoting effects of GH, has become available for clinical investigation. We have initiated a protocol to test the hypothesis that this agent will stimulate growth both in patients with Laron dwarfism, who are resistant to growth hormone due to an absence of functional growth hormone receptor, and in children with non-GH-deficient short stature, who have variable responses to growth hormone that in some cases resemble those of Laron dwarfs.

Since the metabolic effects of growth hormone and IGF-1 on glucose metabolism act in opposing directions, we hypothesize that the combination of these agents might have fewer adverse metabolic effects than either agent alone. Additionally, evidence for synergistic effects of growth hormone and IGF-1 in several systems in vitro suggests the hypothesis that they also may have synergistic effects upon epiphyseal growth in vivo. Thus, we envision, once the effects of IGF-1 alone have been determined, additional studies to test the hypothesis of a synergistic effect of these agents upon linear growth.

3. Differential diagnosis of delayed puberty

No satisfactory diagnostic test is available to determine whether a child with delayed puberty has hypogonadotropic hypogonadism and will never enter puberty spontaneously or has constitutional delay of puberty and will eventually enter puberty on his own. The recent observation that cholecystokinin can stimulate LHRH secretion suggests the hypothesis that children with hypogonadotropic hypogonadism, who lack normally functioning LHRH neurons, will fail to respond to cholecystokinin, whereas children with constitutional delay of puberty will respond normally.

4. Treatment and molecular mechanism of hypoparathyroidism

To test the hypothesis that synthetic parathyroid hormone 1-34 (PTH) could maintain normal serum calcium in hypoparathyroid patients with reduced urine calcium compared to conventional treatment with calcitriol, Karen Winer performed a randomized, cross-over trial comparing these two agents. As we had predicted, PTH permitted equivalent control of serum calcium while achieving a significant reduction in urine calcium. Based upon these findings, we have extended this pilot study to test the hypothesis that PTH treatment will reduce the incidence of renal complications (such as nephrolithiasis, nephrocalcinosis, and decreased renal function) in this disorder.

In the course of this clinical trial, we received referrals of several families with autosomal dominant hypoparathyroidism. A noteworthy feature of these families was that they all had impressive hypercalciuria, even when serum calcium was below the normal range. Following the cloning of the calcium-sensing receptor in December 1993, we hypothesized, based upon our studies of familial male precocious puberty, that the mechanism of this disorder would be an activating mutation of the calcium-sensing receptor. Jeffrey Baron showed this to be the case. One family had a mutation in the transmembrane domain region, which presumably activates signal transduction. Another family had a mutation in the extracellular domain, where it may act to increase the receptor affinity for calcium. Additionally, we found de novo activating mutations of the calcium-sensing receptor in two

children with sporadic hypoparathyroidism and marked hypercalciuria.

The activating mutations of the calcium-sensing receptor predicted a previously unrecognized phenotype of marked hypercalciuria in autosomal dominant hypoparathyroidism. Since the calcium-sensing receptor is expressed in kidney, where it is believed to negatively regulate calcium reabsorption, an activating mutation of this receptor would be expected to cause hypercalciuria even when serum calcium was normal or low. Direct comparison of urine calcium in patients with inherited and acquired hypoparathyroidism showed that the patients with the autosomal dominantly inherited form had significantly greater urine calcium excretion at any given serum calcium level. Moreover, they did not reduce urine calcium as serum calcium decreased, in contrast to the patients with acquired hypoparathyroidism.

Significance to Biomedical Research and the Programs of the Institute:

Growth and puberty are fundamental developmental processes. Growth failure (stature more than 2 standard deviations below the normal height for age) affects approximately 1.5 million American children below the age of 18. Severe growth failure (stature more than 3 standard deviations below normal) affects approximately 100,000 children. Particularly for this severely short group, growth failure may have adverse consequences for the individual's psychological, social, educational, and career development. Thus, improved understanding and treatment of growth failure would benefit a substantial number of children in this country and throughout the world. Additionally, progress in understanding the molecular mechanisms of epiphyseal growth may have implications for understanding the fundamental processes of growth and differentiation in other tissues. Finally, the production of both human growth hormone and insulin-like growth factor-1 by recombinant DNA technology will continue to cause an increasing empiric use of these and other growth-promoting agents in an attempt to increase the adult height of children with growth failure. Thus, the safety and efficacy of growth-promoting agents in this setting has become a public health issue that requires controlled clinical trials.

Precocious puberty occurs in approximately 1 in 5,000 children and often has a profound physical and emotional impact on the affected individuals. The above studies have been highly successful in controlling central and peripheral precocious puberty, disorders for which there has previously been no effective treatment. Progress is also being made in understanding the pathophysiology of the McCune-Albright syndrome and familial male precocious puberty, and in understanding normal human pubertal physiology. Additionally, children with precocious puberty have provided a unique model in which to analyze the influence of hormonal, somatic, and social-environmental factors on the behavioral changes of puberty, which are of importance both to the individual and to society.

Hypoparathyroidism is a rare but potentially fatal disorder that may be acquired, through autoimmune destruction or inadvertant surgical removal during thyroid or parathyroid surgery, or inherited. Although rare, hypoparathyroidism represents a unique opportunity for the scientific study of the role of parathyroid hormone in bone and calcium metabolism. The knowledge gained through such study may have implications beyond this particular disorder, such as to patients with osteoporosis or other metabolic bone disorders. These other disorders represent a major public health problem for which improved understanding of parathyroid hormone effects on bone could be of therapeutic importance.

Proposed Course:

1. The molecular mechanism of familial male precocious puberty (FMPP) and Leydig cell hypoplasia

a. DNA will be obtained from additional affected kindreds. PCR and DNA sequencing of the LH receptor gene will be performed to determine which mutations other than those that are currently known can give rise to these disorders, since each new mutation provides information with which to construct an increasingly precise model of the structural basis for LH receptor activation.

2. Treatment of peripheral precocious puberty

a. Children receiving testolactone treatment of McCune-Albright syndrome and combined testolactone/spironolactone treatment of familial male precocious puberty will be followed until adult height has been achieved. Such follow-up is essential to determine whether the apparent early benefits of treatment will be sustained and whether any significant adverse effects will emerge.

b. Improved therapy for peripheral precocious puberty - the aromatase inhibitor (testolactone) employed in our studies was chosen because of its excellent long-term safety record. Testolactone, however, is not as potent an aromatase inhibitor as would be desirable. More potent aromatase inhibitors are undergoing clinical development. Thus, new patients with FMPP will be randomized to receive fadrozole (CGS 16949A) or testolactone in a crossover design, since fadrozole is approximately 500 times more potent than testolactone. Fadrozole will also be used to treat girls with McCune-Albright syndrome who are refractory to testolactone.

Additionally, our long-range objective is to intercept the pathophysiology of FMPP at the molecular level. Improved knowledge of mechanism could lead to therapies directed at preventing gonadal activation, such as gene therapy or the development of inverse LH agonists capable of restoring basal function in constitutively activated LH receptors, rather than the current strategy of blocking the consequences of gonadal activation.

3. LHRH analog therapy of central precocious puberty

The children currently receiving treatment will be seen annually or biannually until age 11 or 12, at which time treatment will be discontinued. Thereafter they will be seen annually (annual follow-up will be used for the subset of patients who are of greatest research interest because of long duration of treatment and/or proximity to attaining adult height) or biannually until final height is achieved. We plan to maintain an up-to-date address register that will permit subsequent assessment of reproductive function and normalcy of offspring.

4. Optimization of prepubertal and pubertal growth

a. Turner syndrome

We will continue the long-term, randomized, double-blind, placebo-controlled trial to assess the effect on adult height of low-dose estrogen, growth hormone, and the combination of estrogen and growth hormone.

b. Pubertal delay as a treatment to enhance adult height

The ongoing randomized, double-blind trial of LHRH analog compared to placebo will be continued. The trial is fully enrolled (50 subjects), and the children are now being followed until adult height is achieved.

c. Pharmacologic growth hormone treatment in non-growth hormone-deficient in short stature

We will continue the long-term, randomized, double-blind, placebo-controlled trial of the effect of growth hormone on adult height in children with non-growth hormone-deficient short stature.

d. The role of growth factors in epiphyseal growth

We will evaluate whether or not the growth in children with GH insensitivity and with non-GH-deficient short stature can be stimulated with recombinant human insulin-like growth factor-1 (rhIGF-1).

The potential importance of direct administration of growth factors, compared to administration of growth hormone (GH) to stimulate growth factors endogenously, is three-fold. First, it may provide a new approach to treatment of patients with Laron dwarfism, who have resistance to GH and do not respond to GH treatment. It may also be useful in treating patients with familial deletion of the GH gene, who lack tolerance to GH and develop growth-attenuating antibodies to GH during GH therapy. Secondly, we postulate that there may be

negative feedback events that restrain the response to exogenous GH. We hypothesize that rhIGF-1 may bypass such regulatory events, permitting greater responses than could be achieved through GH administration. Additionally, there is evidence in several settings of synergistic interaction between GH and IGF-1. Thirdly, the effects of GH and IGF-1 on glucose metabolism are opposite, which suggests the hypothesis that the combination of these agents may produce less alteration of glucose homeostasis than either agent alone.

We will examine hormonal regulation of epiphyseal TGF- β , FGF, platelet-derived growth factor, growth hormone receptor, and other factors and their receptors, by the techniques of immunostaining, in situ hybridization, solution hybridization, and Northern analysis. These factors will also be examined in vivo by administering them or removing them through classical physiologic approaches in rabbits or through the development of appropriate transgenic mice.

5. Improved differential diagnosis of delayed puberty

Since cholecystokinin has been shown recently to stimulate LHRH release, we will study whether cholecystokinin can be used to assess LHRH secretion in man. The test will be used to examine the hypothesis that idiopathic hypogonadotropic hypogonadal patients will not respond to cholecystokinin, whereas subjects with constitutional delay of puberty will respond.

6. Treatment of hypoparathyroidism

We have conducted a pilot study to assess the hypothesis that calcium metabolism in hypoparathyroidism can be normalized with single daily subcutaneous injections of synthetic parathyroid hormone. The results of the initial phase of the study have confirmed this hypothesis. A short-term, follow-up study is now comparing the safety and efficacy of a once-daily versus a twice-daily PTH regimen. Once the optimal regimen is defined, we plan to conduct a long-term randomized, parallel study to test the hypothesis that PTH treatment can reduce the incidence of renal complications compared to calcitriol treatment.

Protocols:

Animal

92-023	Laue	Nutritional regulation of growth and insulin-like growth factor mRNA expression in rabbit liver, skeletal muscle, and growth plate
94-006	Yanovski	Effects of retinoic acid on rat epiphyseal growth plate

Human

79-CH-112	Cutler	Treatment of true precocious puberty with a long-acting luteinizing hormone releasing factor analog
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This study tests the hypothesis that the growth and pubertal development of children with LHRH-dependent precocious puberty can be normalized by treatment with deslorelin, a long-acting LHRH analog. The study employs an open design in which the major outcomes are compared with the subjects' pre-treatment baseline values (for example, final adult height is compared with predicted height before treatment and hormone levels during treatment are compared with the levels before treatment). The final height data are also being compared with the subjects' target height, the height of the normal population, and the height of untreated historical controls from the literature.

The current results indicate that deslorelin treatment reduces sex steroid levels to near prepubertal values, decreases growth rate and the rate of bone maturation, and produces final heights that are intermediate between the pretreatment predicted height and the children's target height based upon their genetic background. We

conclude that the treatment is effective. Observation is ongoing to determine the adult height of those children who were recognized and treated earliest (who may have a better outcome), to assess the children's reproductive outcome (and thus the reproductive toxicity, if any), and to suppress secondary LHRH-dependent precocious puberty in children who are participating in treatment trials for McCune-Albright syndrome, familial male precocious puberty, and congenital adrenal hyperplasia (see below).

82-CH-165 Feuillan Testolactone treatment of girls with LHRH analog-resistant precocious puberty due to autonomous, non-neoplastic ovarian estrogen secretion

This study tests the hypothesis that the growth and development of girls with the McCune-Albright syndrome can be normalized by treatment with the aromatase inhibitor, testolactone. The long-term portion of the study employs an open design in which the outcome measures are compared with the subjects' pretreatment baseline values and the normative values for control subjects.

The results to date indicate substantial improvement in the majority of subjects, with decreased frequency or cessation of menses and decreased rates of growth and bone maturation. Some patients appear to escape from the effects of treatment after being effectively controlled for 2 to 3 years. This escape is not attributable to secondary central precocious puberty. We conclude that testolactone is a safe and effective treatment for the precocious puberty of McCune-Albright syndrome; however, normalization of growth and development have not yet been achieved, and more effective therapy is needed.

83-CH-199 Yanovski A double-blind, randomized, placebo-controlled clinical trial of luteinizing hormone releasing hormone analog (LHRH A) in pubertal patients with extreme short stature

This study tests the hypothesis that delay of puberty for 4 years by treatment with the LHRH analog, deslorelin, will increase the adult height of children with non-GH-deficient short stature and a normally timed puberty. The design is a randomized, double-blinded, placebo-controlled clinical trial. All 50 subjects have been enrolled.

Final outcome measures are not available because the trial is ongoing. A preliminary, interim analysis early in the trial (after the first 16 subjects had completed the 4 years of LHRH analog or placebo) showed a significant increase in the predicted height of the LHRH analog-treated subjects by 7.6 cm compared to their own pretreatment baseline, and by 10.3 cm compared to the placebo-treated patients. We conclude that pubertal delay induced by deslorelin significantly increases predicted adult height in adolescents with short stature and a normally timed puberty. Whether deslorelin will increase the final height of these patients cannot be determined until they have stopped growing.

91-CH-46 Cutler A randomized, double-blind, placebo-controlled clinical trial of the effects of growth hormone therapy on the adult height of non-growth hormone-deficient children with short stature

This trial tests the hypothesis that supplemental GH administration will increase the adult height of children with non-GH-deficient short stature, without adverse effects owing to supraphysiologic GH dosage. The fundamental rationale for the study is to protect the public health, since thousands of non-GH-deficient children are currently receiving GH treatment before there is convincing evidence of its safety or efficacy in this setting. The design is a randomized, placebo-controlled, clinical trial. 56 of the 80 subjects have been enrolled.

Results of the major endpoints of the study are unavailable because the trial is ongoing. However, the hypothesis that GH decreases thyroid function in some subjects with non-GH-deficient short stature has been examined through a grouped analysis, by an independent statistician, in a manner which preserved the double-blind study design. This analysis showed that supplemental GH, at the dose and frequency employed, did not alter thyroid function during 12 months of treatment in these subjects.

85-CH-16 Cutler Spironolactone and testolactone treatment of boys with familial male

precocious puberty

This study tests the hypothesis that the growth and development of boys with familial male precocious puberty can be normalized by the combination of an antiandrogen, spironolactone, and an aromatase inhibitor, testolactone. The study employs an open design in which the major outcomes are compared with the subjects' pretreatment baseline values and, in the case of growth, with the published standards for the normal population.

The current results, from the initial pilot study, show that the regimen is effective in controlling growth, bone maturation, and pubertal development until secondary LHRH-dependent precocious puberty ensues. At that point, addition of the LHRH analog, deslorelin, can maintain effective control. However, further study is needed to determine the final adult height in the treated patients.

87-CH-152 Cutler A double-blind, randomized, placebo-controlled trial of the effect of biosynthetic growth hormone and/or ethinyl estradiol on adult height in patients with Turner syndrome

This trial tests the hypothesis that supplemental GH treatment, with or without ultra-low, replacement doses of ethinyl estradiol, will increase the adult height of girls with Turner syndrome without adverse effects attributable to the drug regimen. The design is a 4-arm, randomized, placebo-controlled clinical trial, to adult height. The four arms are GH, early (age 5-12) low-dose ethinyl estradiol, GH plus early low-dose ethinyl estradiol, and a double placebo. The starting ethinyl estradiol doses from age 5-8 are 25 ng/kg/d and from age 8-12 are 50 ng/kg/d. However, in recognition of the fact that some girls with Turner syndrome undergo spontaneous puberty, and that sensitivity to estrogen varies among subjects, the protocol provides for dosage decreases of 50% whenever there is breast development below the age of 12 or bone advancement of 1 year within a 6-month period. The dosage can be further decreased by 50% decrements (to 25% or 12.5% of the original dose) if breast development or bone age acceleration persist. Thus, the actual estrogen doses during the prepubertal years in these subjects are extremely small, and are intended to approximate the prepubertal estrogen secretion rate of normal girls. After age 12, all subjects receive the same estrogen regimen beginning at the relatively low dose of 100 ng/kg/day from age 12 to 14, and doubling annually thereafter until menses occur. Approximately 140 of the total 160 subjects have been enrolled.

Major outcome measures from the trial are unavailable because the trial is ongoing. However, analysis of the baseline data from 137 girls with Turner syndrome, before estrogen or growth hormone treatment, revealed significantly increased cholesterol levels after age 11 compared to control girls (195 ± 38 vs. 165 ± 26 mg/dL, $p < 0.01$), which remained elevated after adjustment for body mass index.

This trial, and the Canadian Turner syndrome GH trial, are the only ongoing studies in the world in which the effect of GH in Turner syndrome is being compared to an untreated control group. Thus, the trial will play an important role in resolving this important clinical issue.

90-CH-123 Cutler Estrogen effects on cognition in girls with Turner syndrome

This study tests the hypothesis that the cognitive phenotype of Turner syndrome, namely, decreased performance on tests of spatial recognition, reasoning, and recall, is attributable to estrogen deficiency and will therefore be correctable, at least in part, by estrogen replacement. The study is a companion protocol to project 87-CH-152 in the sense that most of its subjects are also taking part in that study.

Results from this study are not available because of the blinded nature of protocol 87-CH-152. However, the baseline, pretreatment data have confirmed the characteristic features of the Turner cognitive phenotype and have confirmed its stability over time when compared to an untreated adolescent-aged cohort of Turner girls who are not taking part in 87-CH-152. The randomized, placebo-controlled design of this study provides an ideal circumstance in which to test the hypothesis of sex steroid effects on cognitive function, since there is greater control of other variables than has been possible in previous studies.

90-CH-179 Nunez CGS 16949A treatment of girls with precocious puberty due to gonadotropin-

independent ovarian estrogen secretion

This pilot study tests the hypothesis that the new aromatase inhibitor, CGS 16949A or fadrozole, can control the precocious puberty of the McCune-Albright syndrome. The study employs an open design in which the outcome measures during 6 months before treatment are compared with those during treatment. Long-term outcomes, such as adult height, will be compared with the pretreatment predicted adult height, the target height (determined from parental height) and the normal height of the population.

Current results show incomplete blockade of estrogen production at the maximal current dose 240 ug/kg/day. We are currently awaiting FDA approval to evaluate the higher dose of 480 ug/kg/day. If this maneuver is still not fully effective, we plan to test the effectiveness of even more potent aromatase inhibitors once they are available for pediatric use.

91-CH-147	Leschek	Dose-response study and placebo-controlled crossover trial of the effects of IGF-1 therapy on growth velocity in children with GH insensitivity and in children with non-GH-deficient short stature
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This study tests the hypothesis that exogenous IGF-I administration can increase the growth velocity of children with GH insensitivity or with other forms of non-GH-deficient short stature. The study employs a double-blind, randomized crossover design. Results of the study are not available because the trial is ongoing.

91-CH-83	Cutler	A randomized clinical trial of luteinizing hormone-releasing hormone analog in pubertal patients with isolated growth hormone deficiency treated with growth hormone
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This study tests the hypothesis that depot leuprolide-induced pubertal delay will increase the adult height of GH-treated children with classical GH deficiency. The study employs a randomized, open design - all of the children receive GH, and approximately one-half are randomized to receive also the LHRH analog, depot leuprolide. Results are not available since the trial is ongoing and few subjects have attained adult height.

92-CH-011	Winer	Treatment of hypoparathyroidism with synthetic human parathyroid hormone 1-34
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This study tests the hypothesis that once-daily synthetic parathyroid hormone 1-34 (PTH) will normalize serum calcium and reduce the incidence of hypercalciuria, compared to conventional treatment with calcitriol, in patients with hypoparathyroidism. The study employs a randomized, open, crossover design lasting 10 weeks for each arm for the initial dose-adjustment, feasibility phase. Following this initial phase, a second study is testing the hypothesis that twice-daily PTH treatment will normalize calcium metabolism better than once-daily treatment. This second study also employs a randomized, open, crossover design lasting 10 weeks for each arm. Lastly, a long-term study will test the hypothesis that twice-daily PTH will reduce the incidence of renal complications compared to twice-daily calcitriol. This study will employ a randomized, open, parallel design with renal complications (decreased glomerular filtration rate, nephrolithiasis, and nephrocalcinosis) as the major outcome measures.

The current results indicate that PTH, administered once-daily, normalizes serum calcium as well as twice-daily calcitriol while producing significantly lower urine calcium levels at any given level of serum calcium. The preliminary results of twice-daily PTH administration indicate that it normalized calcium metabolism more effectively than once-daily administration. We conclude that PTH offers better short-term control of calcium metabolism than calcitriol in patients with hypoparathyroidism. The effect of long-term PTH administration will require further study.

93-CH-0054	Cutler	The relative effects of androgen, estrogen, and the combination of androgen and estrogen on growth rate, GH binding protein, IGF-I, and cognitive function in Turner syndrome
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This study tests the hypothesis that androgen and estrogen treatment will improve the cognitive function of girls with Turner syndrome in tests of spatial recognition, reasoning, and recall. Additionally, we hypothesize that androgen treatment will have a greater effect on this aspect of cognitive function than estrogen treatment. Lastly, the study tests the hypothesis that androgen and estrogen will have additive effects on growth velocity.

The study employs a randomized, four-arm, open design, with an enrollment goal of 80 subjects. No results are available since the study is still ongoing.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00615-15 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Endocrine-Immune Interactions

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	G.P. Chrousos	Head	SPE, DEB, NICHD
Others:	C.M. Bamberger	Guest Researcher	SPE, DEB, NICHD
	M. De Castro	Guest Researcher	SPE, DEB, NICHD
	D. Papanicolaou	Visiting Associate	SPE, DEB, NICHD
	C. Stratakis	Visiting Associate	SPE, DEB, NICHD
	C. Tsigos	Visiting Associate	SPE, DEB, NICHD
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LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Pediatric Endocrinology

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.8

PROFESSIONAL:

1.6

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to increase our understanding of the interactions between the endocrine and immune systems in both experimental animals and humans. Several immune system products, such as the inflammatory cytokines, Tumor Necrosis Factor- α , Interleukin-1, and Interleukin-6 activate the hypothalamic-pituitary-adrenal (HPA) axis and through it suppress and restrain the inflammatory/immune response. Interleukin-6 is particularly potent in humans, stimulating not only ACTH and cortisol but also arginine-vasopressin (AVP) secretion. Plasma interleukin-6 is elevated in glucocorticoid deficiency states and after exercise. Elevations of interleukin 6 in infectious, inflammatory, and traumatic states may explain the pathogenesis of the Syndrome of Inappropriate AVP Secretion observed in these states. The glucocorticoid antagonist RU 486 potentiated the inflammatory/immune response to a standard inflammatory stimulus in intact animals, suggesting that endogenous glucocorticoids exert anti-inflammatory/immunosuppressive effects at physiological levels. We recently demonstrated that corticotropin releasing hormone (CRH) is produced locally at sites of inflammation and has profound pro-inflammatory effects at an autocrine/paracrine level. We have called this "immune" CRH. Glucocorticoids and somatostatin suppress, and RU 486 markedly augments local secretion of immune CRH at an inflammatory site. Immune CRH was found in the ovary and endometrium, where it may participate in the inflammatory phenomena of ovulation, luteolysis, blastocyst implantation, and menstruation. RU 486 allowed the identification of a central nervous system defect in rats prone to arthritis. In these animals the glucocorticoid response to stress-mediators is inadequate to restrain the immune system following an inflammatory insult. The actual defect is global and located at the level of the hypothalamic CRH neuron, which responds poorly to all its known stimulants, including several cytokines, as well as serotonin, acetylcholine and norepinephrine. This pathophysiologic mechanism is novel and of relevance to human arthritis and other autoimmune diseases. Patients with rheumatoid arthritis have defective pituitary-adrenal axis responses to inflammatory stimuli and produce excessive amounts of immune CRH in their inflamed joints. Patients with fibromyalgia have a slight hypofunction of their hypothalamic-pituitary-adrenal axis revealed by CRH testing and measurements of urinary free cortisol excretion. Patients with multiple sclerosis have mild hypercortisolism, which is sustained by chronic hypothalamic AVP rather than CRH hypersecretion. The human CRH gene contains estrogen-responsive elements in its promoter region providing an explanation for the sexual dimorphism in the incidence of autoimmune/inflammatory disease. CRH antagonists may be useful in the treatment of autoimmune/inflammatory diseases. We found elevated levels of AVP in rats prone to arthritis and patients with rheumatoid arthritis. AVP potentiated the inflammatory response of rats, suggesting that this peptide also participates in inflammation.

Project Description:

We are studying the involvement of interleukin-6 (IL-6) in the interactions between the inflammatory/immune response and the endocrine system and its products. We have shown that IL-6 is one of the most potent stimuli of the hypothalamic-pituitary-adrenal (HPA) axis and arginine-vasopressin (AVP) secretion in man. We are employing recombinant IL-6 as a provocative stimulus of the corticotropin releasing hormone (CRH) neuron in humans with potential alterations in the secretion of this neuropeptide.

Given to rats, the glucocorticoid antagonist RU 486 enhances the inflammatory response and causes enlargement of the thymus and spleen, presumably by antagonizing the immunosuppressive effects of endogenous glucocorticoids exerted at physiologic secretion rates.

One of the potential mechanisms by which RU 486 potentiates inflammation is by stimulation of "immune" CRH production at the level of the inflammatory site. We have shown that the latter is a major pro-inflammatory agent in vivo and that its gene is responsive to estrogen, suggesting that it may play a major role in the sexual dimorphism of the inflammatory/immune response and inflammatory/autoimmune disease. The incidence of the latter is much higher in females than males.

RU 486 allowed the identification of a central nervous system defect in Lewis rats, a strain prone to development of autoimmune arthritis and other inflammatory states. In these animals the glucocorticoid response to stress-mediators is inadequate to restrain the immune system following an insult. This pathophysiologic mechanism is novel and of relevance to human rheumatoid arthritis and other inflammatory/autoimmune diseases. We have demonstrated that patients with rheumatoid arthritis have subnormal responses of the hypothalamic-pituitary-adrenal axis to inflammatory stimuli, which can explain their unrestrained inflammatory response in the joints and other tissues.

Objectives:

The objectives of this project are to increase our understanding of the physiologic interactions between the endocrine and immune systems and of the impact of alterations in these interactions on the development of human disease. The knowledge obtained may assist us with improving the diagnosis and treatment of diseases in which these interactions are disturbed.

Methods Employed:

Methods consist of glucocorticoid and other receptor assays, and in vivo tests of glucocorticoid action (thymus involution, glycogen deposition, growth retardation, suppression of inflammation). Radioimmunoassays and/or ELISA assays for measurement of pertinent hormones or cytokines are also used. We have developed a thin layer and high performance liquid chromatography (HPLC) method and a radioreceptor assay procedure to purify and measure RU 486 in biological fluids. We have also cloned human glucocorticoid receptor mutants/isoforms in an expression vector and employ a co-transfection system in which a glucocorticoid-responsive reporter gene is used to study the functional properties of these mutants/isoforms. We use in situ hybridization and Northern blots to measure expression of glucocorticoid, POMC, AVP and AVP receptor and CRH, CRH receptor and CRH binding protein mRNA in the hypothalamus, pituitary, and other tissues. We use standard immunohistochemistry and/or extraction-HPLC-radioimmunoassay to demonstrate presence and distribution of CRH and other neuropeptides and cytokines in tissues. We also employ a hypothalamic organ culture system for examination of CRH and AVP secretion and a dispersed anterior pituitary culture system for examination of ACTH secretion. We employ standard nucleic acid extraction and cloning and sequencing techniques. We have developed our own antibodies against CRH and other peptides, as well as against isoforms of the glucocorticoid receptor, for performing assays, immunohistochemistry, Western blots, and/or immunoneutralization studies.

Progress:Effects of Interleukin-6 in Man

We have performed dose-response studies with recombinant interleukin-6 in humans by administering the cytokine parenterally. Interleukin-6 causes sustained elevations of plasma ACTH, cortisol and arginine-vasopressin, without major side effects, such as hypotension or vascular leak phenomena, as one observed earlier with tumor necrosis factor- α (TNF- α) and Interleukin-1. We have demonstrated that exercise causes an intensity-dependent elevation of plasma IL-6 concentration, which is suppressed by pretreatment of the subjects with glucocorticoids. Patients with glucocorticoid deficiency have elevated plasma concentrations of IL-6. We have demonstrated by cross-correlation analysis of plasma IL-6, ACTH and cortisol concentrations in patients with early, untreated rheumatoid arthritis, that IL-6 has a circadian rhythm with a morning surge preceding that of ACTH and cortisol. We found the IL-6 drives ACTH and cortisol in the morning, but is also suppressed by the latter at later times.

Usefulness of the Glucocorticoid Antagonist RU 486

The availability of RU 486 provided the unique opportunity to study two longstanding questions on the effects of glucocorticoids on the immune system and inflammatory response. First, are these effects glucocorticoid receptor-mediated? And second, do endogenous glucocorticoids exert an effect on the immune system at physiologic secretion rates? In an "aseptic inflammation" rat model, glucocorticoids suppress exudate formation, leukocyte diapedesis and prostanoid accumulation. RU 486 was able to antagonize the effect of pharmacologic doses of glucocorticoids, suggesting that they are receptor-mediated. In addition, RU 486 given alone caused enhancement of the inflammatory response, suggesting that endogenous glucocorticoids at physiological secretion rates exert suppressive effects on the inflammatory response in the rat. RU 486 also caused enlargement of the thymus and spleen in these animals. These promising observations suggested that a glucocorticoid antagonist could be used as an immune enhancer in conditions requiring such enhancement (cancer, immunodeficiency).

We recently found that human tissues contain an endogenous non-ligand binding glucocorticoid receptor isoform, which acts a dominant negative inhibitor of the glucocorticoid receptor. This inhibitor may define the sensitivity of the immune system to glucocorticoids. Increased expression might cause glucocorticoid resistance and, hence, a hyperactive immune/inflammatory reaction. Decreased expression might cause glucocorticoid hyposensitivity and hence, immunosuppression. Whether this isoform is involved in the pathogenesis of autoimmune/inflammatory disease is examined under project Z01 HD-00618-14-DEB.

RU 486 was instrumental in identifying a central nervous system defect in rats prone to arthritis in response to antigenic stimulation with streptococcal cell wall polysaccharide (SCW). These rats (Lewis rats) had deficient CRH, ACTH and glucocorticoid responses to stressors, including inflammatory stimuli. "Histocompatible" rats (Fischer rats), which normally do not develop arthritis in response to SCW, do so when treated with RU 486. We defined the CNS defect of Lewis rats in the hypothalamic CRH neuron, which is globally underresponsive to the inflammatory cytokines, and the stimulatory neurotransmitters serotonin, acetylcholine and norepinephrine. We believe the defect is in the 5' regulatory region of the CRH gene or more likely in one of the related transcription factors (see specific section below).

"Tissue" CRF's vs. "Immune" CRH

The presence of non-hypothalamic "tissue" CRFs was suggested by Brodish in the early 70's. The concept was revived by Woloski et al. in 1985. We have employed a specific antibody to prove both the existence of an immunoreactive "tissue" CRH and its importance for the immune/inflammatory reaction in vivo, in the whole animal. This is separate from the various humoral mediators of inflammation, such as the inflammatory cytokines TNF- α , interleukin-1 and interleukin-6, or lipid mediators of inflammation, such as several eicosanoids and platelet activating factor (PAF), which stimulate CRH neuron function and/or pituitary ACTH secretion in vitro.

and/or in vivo.

A number of recent reports suggested that the interactions between the nervous, endocrine and immune system were more extensive than those mentioned above. These recent studies presented evidence that CRH had direct effects upon leukocytes. For example, CRH directly stimulated production of proopiomelanocortin (POMC)-related peptides and secretion of interleukin -1 and -2, stimulated lymphocyte proliferation, enhanced the proliferative response of leukocytes to lectins and increased the expression of the IL-2 receptor on T lymphocytes. These reports were complemented by the finding of specific CRH binding sites in various subpopulations of leukocytes and, finally, by presence of CRH immunoreactivity and CRH mRNA by *in situ* hybridization and Northern blotting, in resting subpopulations of human leukocytes. All these data suggested strongly that CRH might have local direct effects upon the immune/inflammatory system. We attempted to identify the potential biological role of these effects by first examining the ability of systemic CRH immunoneutralization to influence the size of a quantifiable inflammatory response. As such, we employed the aseptic subcutaneous reaction to carrageenin.

We studied male Sprague-Dawley rats that were injected intraperitoneally with neutralizing rabbit anti-CRH antisera one hour before subcutaneous injection of carrageenin. Both exudate volume and cell concentrations were suppressed by approximately 50-60%, whereas no such suppression was observed in control rats pretreated with normal saline, normal rabbit sera or anti-TSH rabbit antisera. The specific suppression of the inflammatory response observed after CRH immunoneutralization was clearly opposite to what could be expected from abrogation of hypothalamic-hypophyseal portal CRH. The latter should have resulted in relative hypoglucocorticoidism and enhancement of the inflammatory response, as is seen in animals pretreated with a glucocorticoid receptor antagonist. These findings are instead compatible with the view that local effects of CRH are proinflammatory. This concept was complemented by two different sets of data. First, parallel experiments with TNF- α neutralizing antisera also showed marked suppression of inflammation, approximately of the same magnitude as that observed with anti-CRH antisera. TNF- α is a major auto/paracrine proinflammatory agent, which directly, or via IL-1 and/or IL-6, activates the inflammatory response. No additive effect was observed between anti-CRH and anti-TNF, a finding compatible with common or shared mechanisms of inflammation suppression between these two antisera. Second, direct local administration of anti-CRH antisera into the air pouch also decreased the inflammatory reaction.

Since the above findings suggested that CRH might participate in the inflammatory process in vivo as a local stimulatory agent, we examined the site of inflammation for evidence of CRH production. Several approaches were taken. First, significantly high amounts of immunoreactive CRH were intracellularly detected in the inflamed tissues by specific immunohistochemistry, using an affinity-purified anti-CRH antibody. Second, direct measurement of CRH immunoreactive content of extracted inflammatory tissue demonstrated a high quantity of CRH immunoreactivity at the range of 400pg/g of wet tissue. High CRH concentrations have also been demonstrated in the rat hypothalamus and human placenta. Sizing of the CRH-immunoreactive molecule by HPLC revealed a fraction eluting at the location of rat/human CRH 1-41, suggesting that CRH acting in inflammatory sites, designated 'immune CRH', is similar in chromatographic mobility to that produced by the hypothalamus and the human placenta. These findings, taken together, strongly suggest that there is a local production of a CRH-like molecule by inflammatory tissues, whose immunoneutralization results in decrement of inflammation. The tissue concentrations of this molecule are of sufficiently high concentrations to produce biological responses, including POMC-derived peptide secretion and regulation of immune functions. We have demonstrated that exogenous glucocorticoid or somatostatin suppress immune CRH secretion in a dose-dependent fashion, in parallel to the suppression of the inflammatory/immune response.

To examine whether immunoreactive CRH produced locally, at the inflammatory site, might have distant, endocrine effects, we measured plasma concentrations of IR-CRH in carrageenin- and placebo-treated animals through the 7h ensuing carrageenin or placebo administration. The concentrations of plasma CRH were quite low, at the range of 8-10pg/ml ($\sim 10^{-12}$ M) and similar in the two groups of animals throughout the experiment. These levels are markedly lower than those of the rat hypophyseal portal blood and not expected to be bioactive in vivo

or in vitro. Based on these data we suggest that immune CRH is an auto/paracrine, rather than endocrine, hormone and that rapid metabolism prevents it from reaching biologically significant circulating concentrations. In this sense, it is similar to other autacoids with known major proinflammatory or anti-inflammatory activity, of which platelet activating factor (PAF) and prostaglandin E_2 are examples. Other paradigms of peripheral CRH secretion with primarily auto/paracrine function is its production by chromaffin cells of the sympathetic ganglia and adrenal medulla and by Leydig cells of the testis. In the former it appears to potentiate catecholamine secretion, while in the latter it seems to be involved in an apparent autocrine inhibitory feedback on testosterone production.

The data from our study suggests that CRH plays a dual, antithetical-albeit counterbalanced role in the regulation of the inflammatory/immune response. Indirectly, hypothalamic CRH suppresses this response by activating glucocorticoid secretion and causing increased sympathetic outflow, two phenomena leading to immunosuppression. Immune CRH, on the other hand, has potent proinflammatory actions which can be unmasked by use of neutralizing CRH antisera and, perhaps, peripheral antagonists of this peptide. Does immune CRH have any peripheral roles in addition to its proinflammatory ones? For instance, it may be involved in antinociception by stimulating a known peripheral analgesic, β -endorphin, or by exerting its own direct antinociceptive effects. The latter would be analogous to its recently described antinociceptive effects in the central nervous system. How are the effects of immune CRH exerted on the immune system at a molecular level? Its major second messenger in the pituitary corticotroph is cyclic AMP(4) which has been primarily shown to suppress immune functions when elevated. Would this suggest that immune CRH acts through a different receptor or second messenger system? Many questions have been generated from this study and clinical applications of immune CRH agonists or antagonists as potentiators or inhibitors of the inflammatory/immune response can be envisioned.

Secretion of immune CRH in inflammatory sites appears to be a generalized phenomenon. We extended our observations to several experimental and human states, including retinol-binding protein-induced uveitis in rats and mice, and human rheumatoid arthritis and Hashimoto thyroiditis. Immunoneutralization of CRH and TNF- α ameliorated uveitis in mice only when given in the early phase of the disease, suggesting that both peptides play a major role in the initiation and early propagation of inflammation. Glucocorticoids and somatostatin suppressed immune CRH secretion, in accordance with their antiinflammatory effects. Glucocorticoids, in fact, stimulated somatostatin secretion within the inflammatory sites and antisomatostatin antibodies inhibited in part the antiinflammatory actions of glucocorticoids, suggesting that somatostatin may play the role of an intermediary auto-paracrine messenger.

Immune CRH in Reproductive Tissues

Since both ovulation and luteolysis and blastocyst implantation and menstruation represent aseptic inflammatory phenomena, we considered the ovary and endometrium as potential sites of CRH secretion and action. Indeed we have demonstrated secretion of CRH in both the rat and human ovary as well as the presence of specific CRH receptors in these gonads. The theca and stroma as well as the corpora lutea contained both. We are currently studying the role(s) of CRH in the ovary along with its second messenger system in ovarian cells. We found decreased CRH expression in the theca and stroma of human polycystic ovaries. We also found that CRH appears to inhibit steroidogenesis by cultured luteinized granulosa cells. This suggests that androgen hypersecretion by polycystic ovaries may be related to decreased inhibition by CRH. Recently, we also demonstrated CRH in the glandular epithelium of the human endometrium at both phases of the cycle, and within the endometrial stromal cells and local macrophages in the luteal phase.

A Central Nervous System Defect Linked to Autoimmune/Inflammatory Disease

We have demonstrated that the propensity of Lewis rats to develop severe inflammatory arthritis in response to streptococcal cell wall polysaccharide (SCWP) is related to their inability to mount an adequate counter-regulatory activation of their HPA axis during the inflammatory response. "Histocompatible" Fischer rats, which normally

do not develop arthritis in response to SCWP, do so, when treated with the glucocorticoid antagonist RU 486. The diminished HPA axis response in Lewis rats appears to result from a generalized defect in the synthesis and secretion of hypothalamic CRH in response to different types of physiologic and pharmacologic stimuli. We believe that a defect in the CRH neuron of Lewis rats, which makes it globally under-responsive to IL-1, serotonin, acetylcholine norepinephrine and other physiologic regulators, is responsible for the hyposecretion of glucocorticoids during the immune/inflammatory response. Interestingly, the Sprague-Dawley rat, from which the first histocompatible Lewis and Fischer rats were bred, has an intermediate position between the two other strains in both HPA axis activity and propensity to develop autoimmune inflammatory disease in response to SCWP. We have studied in detail the basal and stress-induced pattern of ACTH and corticosterone secretion in the Lewis and Fischer rats. A blunted circadian rhythm, with lowered time-integrated ACTH and corticosterone secretion, and hyporesponsiveness to any kind of stress (inflammatory, immobilization, and other) characterizes the Lewis rat. We extended the original findings with SCWP to another form of inflammation, the carrageenin model - with similar differences between the strains. Interestingly, although the hypothalamic CRH response was decreased in Lewis rats compared to Fischer rats, copious amounts of IR-CRH were found in the joints and carrageenin inflammation sites of the former. This suggests that both lack of glucocorticoid-induced suppression and excess of local CRH secretion in the Lewis rat may be responsible for the excessive inflammatory response observed in this strain, and that the regulation of CRH synthesis/secretion is different between the CNS and the periphery. The latter could be explained by different tissue-specific regulation or be deficient local glucocorticoid-mediated suppression of immune CRH synthesis/secretion. Interestingly, Lewis rats had elevated plasma levels of arginine vasopressin (AVP) compared to Fischer rats. Immunoneutralization of AVP ameliorated carrageenin-induced inflammation, suggesting that AVP participates in inflammation as a proinflammatory agonist. The Lewis and Fischer rats have another fascinating property, when compared to the maternal Sprague-Dawley rat strain. The former has behaviors characteristic of low central nervous system (CNS) CRH, whereas the latter has behaviors typical of high CNS CRH. These strains represent, thus, interesting models of stress system hypo- and hyperfunction, reminiscent of the data described in atypical and melancholic depression, respectively.

The major question comes up whether the above findings bear any relevance to human pathophysiology. We have demonstrated presence of high levels of IR-CRH with the chromatographic mobility of hCRH 1-41 on HPLC in synovial fluid and histologic specimens from joints of patients with rheumatoid arthritis. Severe behavioral problems in such patients have been noted, reminiscent of atypical depression, and the question as to whether a subgroup of human patients with rheumatoid arthritis or other autoimmune inflammatory diseases have inadequate central "endocrine" counter-regulatory responses upon the immune/inflammatory response, combined with behavioral traits related to changes in the regulation of the HPA axis, is actively being pursued. We have evidence that patients with rheumatoid arthritis are indeed hyporesponsive to inflammatory stimuli, having thus pathophysiologic mechanisms similar to those observed in the Lewis rat. We are planning to study this further by employing recombinant interleukin-6 (IL-6) as a test stimulus in patients with autoimmune diseases or vulnerability to such diseases.

We studied the HPA axis of patients with fibromyalgia and multiple sclerosis. The former had evidence of hypofunction of this axis, similarly to patients with chronic fatigue syndrome. The latter had mild chronic hypercortisolism, which however was sustained by high hypothalamic AVP rather than CRH secretion. We also studied the HPA axis of postpartum women, given that this period is characterized by increased incidence of autoimmune/inflammatory diseases. We found transiently suppressed hypothalamic CRH secretion in these patients, which would explain their increased vulnerability to such diseases (please see project Z01 HD-00618-14-DEB).

Significance to Biomedical Research and the Program of the Institute:

Interleukin-6 is a very potent and relatively innocuous stimulus of the HPA axis in man. We have shown that it carries promise as a diagnostic tool and as a probe to study adrenal physiology. As a challenger of the hypothalamic-pituitary-adrenal axis it holds promise in the study of disorders of the axis: Cushing's syndrome, adrenal insufficiency, psychiatric hypercortisolism and hypocortisolism and vulnerability to psychiatric and

autoimmune disease. As an experimental tool, RU 486 provides an excellent means to study stress physiology and the role of glucocorticoids upon the immune system and the inflammatory response. Recently, RU 486 gave us new insight on the pathogenesis of arthritis and/or other inflammatory/autoimmune diseases. In these studies, CRH was found to be a major local cytokine, influencing positively the inflammatory response. The CRH gene was found to be responsive to estrogen, suggesting that the female-male differences in the inflammatory/immune response and the incidence of inflammatory/autoimmune disease might be explained by sexual dimorphism in the regulation of this gene. CRH was found to participate in ovarian and endometrial functions, probably promoting physiologic aseptic inflammatory processes. Preliminary studies suggest that it may also play a role in blastocyst implantation.

Proposed Course:

- 1) We have used interleukin-6 as a new provocative test of hypothalamic-pituitary-adrenal function. We are now completing studies with psychiatric hypercortisolism (melancholic depression, pseudoCushing) and hypocortisolism (atypical depression, chronic fatigue-fibromyalgia, postpartum blues/depression) and autoimmune/inflammatory disease (rheumatoid arthritis, Hashimoto thyroiditis), conditions in which the pituitary ACTH reserve or the glucocorticoid negative feedback may be significantly altered. Of special interest would be the detection of individuals premorbidly, to define who is vulnerable to develop a disease characterized by a hyperresponsive (melancholic depression, panic anxiety) or hyporesponsive (atypical depression, chronic fatigue/fibromyalgia, postpartum blues/depression, autoimmune/inflammatory diseases) HPA axis.
- 2) We are now defining the anatomic locus and the molecular mechanisms of the defective HPA axis response of Lewis rats. It appears that there is generalized deficiency of CRH neuron responsiveness to various stimuli. We plan to clone the 5' regulatory region of the CRH gene in these animals, to sequence it and to examine its functional activity in the presence of Lewis and Fischer rat hypothalamic nuclear extracts.
- 3) It is of utmost importance to define the actual cellular target(s) of immune CRH, to demonstrate the presence of CRH receptors in these tissues, to identify the type of receptors (CRH-R1, -R2 α or -R2 β) present and to show whether the recently characterized CRH-responsive element-binding protein is present in such target cells. We plan to employ newly developed CRH antagonists to explore the therapeutic potential of these compounds in inflammatory states as topical antiinflammatory drugs or as anti-edema/anti-vascular permeability agents.
- 4) We plan to examine the potential functional significance of immune CRH in reproductive tissues, using newly developed potent CRH antagonists. We have a particular interest in the phenomenon of blastocyst implantation, an aseptic inflammation-like process.

Protocols:

Human

82-CH-45	Chrousos	Dose-Response Relationships for Single Doses of Corticotropin-Releasing Hormone (active)
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This blanket protocol enables us to administer ovine corticotropin-releasing hormone (CRH) as a provocative test of the hypothalamic-pituitary-adrenal (HPA) axis in men, women, and children with potential alterations of this axis. The peptide continues to be an investigational drug (IND# 19802) and all data concerning its administration are captured and reported annually to the FDA. During the past year oCRH was administered under this and other approved protocols.

88-CH-120	Chrousos	The Hypothalamic-Pituitary-Adrenal Axis in Pregnancy, the Postpartum Period and in Postpartum Depression Syndromes (precis and brief analysis included in project Z01 HD-00618-14-DEB)
94-CH-134	Papanicolaou	Dose-Response Relationships for Single Doses of Recombinant Interleukin-6 in Normal Volunteers and Patients with Disorders of the Hypothalamic-Pituitary-Adrenal (HPA) Axis
		This protocol concerns the administration of recombinant interleukin-6 to normal volunteers and patients with disorders of the HPA axis. Interleukin-6 is an investigational drug (IND# 5419) with which we have already obtained some experience from a phase 1 study of cancer patients done by NCI. A dose-response curve in normal volunteers has been completed and a dose has been selected to be administered to the various patient groups listed in the protocol. The hypothesis of the protocol is that as a hypothalamic stimulus of CRH, acute administration of interleukin-6 will distinguish biochemically not only patients with a clearly disordered HPA axis, i.e. Cushing's <u>vs.</u> pseudoCushings's <u>vs.</u> melancholic depression <u>vs.</u> chronic active alcoholism or melancholic <u>vs.</u> atypical depression, but also premorbid states of vulnerability, i.e. individuals prone to melancholic depression or anxiety disorders or individuals prone to atypical depression, chronic fatigue/fibromyalgia, postpartum blues or depression, alcoholism or autoimmune disorders.

Animal Protocols:

89-036 rat	Chrousos	Regulation of Hypothalamic CRH and Pituitary ACTH Release (active)
91-036 rat	Chrousos	Dose-Response Studies Between Dexamethasone and Suppression of Thymic and Adrenal Weights in Fischer and Lewis Rats (completed)
92-032 rat	Webster	Reproductive Tissue Inflammation-like Processes and Local CRH Production (active)
95-014 rat	Chrousos	Role of Corticotropin Releasing Hormone in Implantation (active)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00616-14 DEB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Structure, Function, and Physiology of Glycoprotein Hormones

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. M.R. Flack Senior Clinical Investigator SME, DEB, NICHD

Others: B.C. Nisula Head SME, DEB, NICHD
 D. Blithe Expert SME, DEB, NICHD
 J. Anasti Clinical Associate SME, DEB, NICHD
 J. Froehlich Visiting Fellow SME, DEB, NICHD
 C. Finch Biotechnician SME, DEB, NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Medical Endocrinology Section

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

4.0

PROFESSIONAL:

3.5

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00618-14 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physiology and Pathophysiology of the Hypothalamic-Pituitary-Adrenal and Gonadal Axes

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: G.P. Chrousos Head SPE, DEB, NICHD

Others: See attached list.

COOPERATING UNITS (if any)

See attached list.

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Pediatric Endocrinology

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

8

PROFESSIONAL:

6.6

OTHER:

1.4

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

In this project we seek to advance the understanding of the physiology and pathophysiology of the hypothalamic-pituitary-adrenal (HPA) and -gonadal (HPG) axes. The role of stress-related hormones in normal and disease states is being examined, and clinical applications for these hormones are sought. The discovery of the structure of corticotropin releasing hormone (CRH) and the development of sensitive assays for measuring HPA- and HPG-related hormones and their receptors have led to rapid progress in this field. Major progress has been made in three areas: 1) Clinical applications of CRH: An ovine (o) CRH stimulation test has been developed that is useful in the differential diagnosis of adrenal insufficiency, Cushing's syndrome, and pseudo-Cushing's states (psychiatric hypercortisolism). The oCRH test and/or measurements of CSF CRH have increased our understanding of the pathophysiology of Cushing's syndrome, melancholic depression, childhood sexual abuse, atypical/seasonal depression, the chronic fatigue/fibromyalgia syndromes, diabetes mellitus, rheumatoid arthritis, and the postpartum blues/depression syndromes. 2) The regulation of the axis by neurotransmitters, neuropeptides, and glucocorticoids has been studied in vivo and/or in vitro. Third trimester pregnant women and athletes have a hyperfunctional pituitary-adrenal axis in the resting state. The hCRH gene 5' regulatory region has been cloned and sequenced and its regulation has been studied. It has 2 active promoters and responds positively to estrogens, providing a potential explanation for the sexual dimorphism of psychiatric diseases characterized by aberrations in CRH secretion. 3) Roles and actions of HPA and HPG axes hormones. Glucocorticoid resistance is an autosomal recessive or dominant disease associated with abnormalities of the glucocorticoid receptor. We have elucidated the molecular pathophysiology of this syndrome by defining mutations and/or deletion of the glucocorticoid receptor gene leading to abnormal or decreased receptors in the tissues of patients. The mineralocorticoid receptor and the subunits of the amiloride-sensitive sodium channel are studied in sporadic cases of pseudohypoaldosteronism or mineralocorticoid resistance. The interaction of the classic glucocorticoid receptor (GR α) and its nonligand binding natural homolog glucocorticoid receptor β (GR β) with each other and with the heat-shock proteins and glucocorticoid-responsive elements (GREs) of the DNA are studied, as the well as the importance of GR β in human physiology and pathophysiology. We have elucidated the molecular pathophysiology of hereditary ACTH resistance, an autosomal recessive disorder characterized by isolated glucocorticoid deficiency, by defining abnormalities of the ACTH receptor gene. We have elucidated the molecular pathophysiology of testicular and ovarian resistance to luteinizing hormone (LH) by defining abnormalities of the LH receptor gene. We have localized the gene for Carney Complex, a multiple neoplasia/lentiginosis syndrome, on chromosome 2p16.

Professional Personnel: (continued)

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	G. Cizza	Visiting Associate	SPE, DEB, NICHD
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	A.C. Latronico	Guest Researcher	SPE, DEB, NICHD
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	C. Tsigos	Visiting Associate	SPE, DEB, NICHD
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Project Description

Objectives:

The term stress encompasses the sum of body reactions that take place during the influence of stimuli (stressors) threatening to alter homeostasis. The changes occurring are both behavioral and physical. Stress is associated with activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic system. It appears that these two systems interact at various levels, from the CNS to the peripheral target tissue, and when activated have profound effects on other systems, including those subserving reproduction, growth and immunity. The structure of CRH, the principal hypothalamic hormone regulating pituitary-adrenal function, was discovered in 1981 after over 20 years of investigation. This discovery made it possible to begin to explore the role of this hormone in stress and in human health and disease. The general objectives of this project have been to understand the physiologic and pathophysiologic mechanisms of stress in experimental animals and man and to study the function of the hypothalamic-pituitary-adrenal axis in stress, health and disease. For clarity, we have divided the "Progress", "Significance to Biomedical Research and the Program of the Institute" and "Proposed Course" sections of the annual report into three broad sections: a) Clinical Applications and Implications of CRH, b) Regulation of the Hypothalamic-Pituitary-Adrenal Axis In Vivo and In Vitro, c) Roles, Actions and Pathophysiology of HPA and Hypothalamic-Pituitary-Gonadal (HPG) Axes Hormones, and d) Mechanisms of Adrenocortical Tumorigenesis.

Methods Employed:

- 1) Metabolic clearance studies - Methods consist of (1) radioactive labeling and chromatographic purification of a hormone or (2) measurement of that hormone by radioimmunoassay, immunoradiometric assay or enzyme-linked assay and estimation of its metabolic clearance rate by the pulse injection or continuous infusion methods.
- 2) Radioimmunoassay (RIA)/Immunohistochemistry - RIAs have been developed to measure ovine and human CRH, ACTH and POMC-derived peptides such as beta-endorphin and alpha-MSH, as well as other peptides. Also, RIAs have been developed for vasopressin, dynorphin A, tumor necrosis factor- α (TNF- α), δ -sleep-inducing peptide and neuropeptide Y. Affinity purification of antibody and immunohistochemistry procedures for several of the above peptides have been set up.
- 3) Peptide hormone receptor assays have been used to examine the presence and characteristics of these receptors in various tissues or cells.
- 4) An ACTH bioassay in which rat adrenal corticosterone secretion is examined as an end-point.
- 5) A pituitary culture system is being used to examine the CRH activity of various extrahypothalamic substances with CRF bioactivity but not immunoreactivity.
- 6) A hypothalamic organ culture system in which the secretion of CRH from rat hypothalami is examined in vitro. This allows us to directly examine the regulation of CRH neuron function by neurotransmitters, neuromodulators, and feedback systems.
- 7) An organ perfusion system of human or rat full thickness placenta fragments has been established in which we study the secretion of CRH and POMC-derived peptides. The regulation of placental secretion of these peptides is studied.
- 8) POMC, CRH, AVP mRNA assays by Northern blotting, RNase protection, and in situ hybridization are used to determine the site of POMC, CRH, and AVP gene expression as well as their regulation. Similar methods have been established for the CRH and AVP receptors and the CRH-binding protein.

9) Glucocorticoid receptor assays - both binding assays, protein assays by Western blotting and assays of receptor mRNA by Northern blotting or in situ hybridization for both types of the glucocorticoid receptor, glucocorticoid and mineralocorticoid, are performed in our laboratory. The regulation of the glucocorticoid receptor gene expression, especially its alternative splicing to the classic glucocorticoid receptor and its natural nonligand-binding isoform β , is examined. The molecular pathophysiology of glucocorticoid and mineralocorticoid resistance in man and nonhuman New World primates is examined.

10) Standard cloning and sequencing procedures. These are applied in the cloning of the glucocorticoid, mineralocorticoid, ACTH and LH receptor in families or subjects with cortisol, aldosterone, ACTH and LH resistance, and in the cloning of other genes of choice. Co-transfection assays and use of reporter plasmids expressing chloramphenicol acetyl transferase (CAT) or luciferase coupled to appropriate promoters have been developed to assess the functional activity of several gene products.

11) Standard genetic linkage analyses for chromosomal localization of hereditary endocrine disorders, such as Carney complex and the Triple A syndrome.

Progress:

A. Clinical Applications and Implications of CRH

From testing normal subjects it has become evident that age plays no major role in the responsiveness of plasma ACTH and cortisol to CRH in man. Children above five to old men in their nineties have virtually indistinguishable responses. There is a small difference in the responsiveness of the axis between men and women characterized by exaggerated ACTH responses and slight prolongation of the cortisol responses in the latter. There are no changes in the responsiveness of the HPA axis during the different phases of the menstrual cycle in normal women.

CRH causes elevations of plasma ACTH and cortisol in patients with pituitary Cushing's disease, whereas it does not in patients with the ectopic ACTH syndrome. The differential diagnostic accuracy of the CRH test in this condition exceeds 85 percent. Patients with Cushing's syndrome of adrenal etiology have low or undetectable levels of plasma ACTH, that fail to respond to exogenous CRH. Thus, the CRH stimulation test is a useful outpatient, brief, safe and easy diagnostic tool in the differential diagnosis of Cushing's syndrome, as it distinguishes pituitary from adrenal or ectopic causes, each requiring a different therapeutic approach. A prospective study of our patient series suggests that the oCRH test is equal, or superior, in its predictive value than the standard tests which are in-patient, long, and cumbersome.

Pituitary Cushing's disease is usually due to small ACTH-secreting adenomas. Cure is achieved by transsphenoidal adenomectomy. We have improved the cure rate by introducing a new sampling method in association with oCRH administration. We are sampling the veins draining the pituitary gland (petrosal sinuses) simultaneously for measurement of ACTH both basally and after CRH. In the overwhelming majority of patients an ACTH concentration gradient is found between one or both petrosal sinuses and a peripheral vein. Thus, the surgeon searches for a pituitary adenoma. Continuous ovine CRH administration as early as a week after successful, curative surgery stimulates ACTH and cortisol levels into the normal range, suggesting that the profound adrenal insufficiency normally observed in cured patients is of hypothalamic or suprahypothalamic origin.

In adrenal insufficiency, testing with CRH helps differentiate between hypothalamic and pituitary causes, since in the former there are ACTH responses to CRH, whereas in the latter there are not. It is interesting that hCRH, which we have shown to be short-acting and reproducing the spontaneous secretory episodes of ACTH and cortisol, restores, when given in a pulsatile fashion, the normal pattern of ACTH and cortisol in patients with hypothalamic adrenal insufficiency. Glucocorticoid therapy administered on alternate days is associated with a normal ACTH and cortisol response to CRH on the day off. Interestingly, adrenal androgen responses are

suppressed suggesting that alternate therapy may be a good therapeutic option for growing children with hyperandrogenism. Patients with the late-onset form of congenital adrenal hyperplasia had an extensive evaluation of the HPA axis. Interestingly, it appeared that the equilibrium between ACTH and cortisol in these patients as a group was totally normal. We considered this an inherent long-standing adaptation of the HPA axis in these patients.

Patients with psychiatric disorders associated with hypercortisolism (depression, panic anxiety, anorexia nervosa) have decreased plasma ACTH responses to exogenous CRH. The blunting of the response is inversely proportional to the degree of hypercortisolism. Thus, patients with pseudo-Cushing's states could potentially be differentiated from patients with Cushing's disease who respond differently to exogenous CRH. The diagnostic accuracy of the test is approximately 75% in distinguishing these two conditions. Pretreatment of the patients with dexamethasone improves significantly the distinction between the two conditions.

Recently, the role of CRH has been expanded from merely that of an ACTH secretagogue to that of a neuromodulator of broader significance. Intracerebroventricular administration of CRH in rats causes physiological (HPA axis activation, sympathetic activation, hypogonadism) and behavioral (aggression, irritability, anorexia, loss of libido) changes that are normally observed in stress. These findings have suggested a pathophysiologic role of CRH in psychiatric conditions characterized by chronic hypercortisolism. If this hypothesis is correct, treatments directed towards preventing endogenous CRH hypersecretion or CRH antagonists may be developed for these conditions.

We compared the diagnostic utility of hCRH to that of oCRH in Cushing's syndrome and psychiatric hypercortisolism. The ovine CRH analog is clearly superior to the human one for diagnostic testing. However, the hCRH analog will remain useful in studies requiring restoration of the physiologic pulsatile pattern of ACTH and cortisol.

We have immunized rabbits with CRH and have produced antisera against both ovine and human CRH. We have used these antisera to produce immunoneutralization *in vivo* or to develop radioimmunoassays for these peptides. These radioimmunoassays are sensitive to the pg/ml level and have been extensively used to estimate the metabolic clearance rates of these peptides in normal men and patients with adrenal insufficiency, Cushing's syndrome, depression, and anorexia nervosa. Strong stress stimuli, such as surgery, exercise, or insulin-induced hypoglycemia, fail to cause major elevations of plasma CRH immunoreactivity. CRH immunoreactivity can be readily measured in the cerebrospinal fluid (CSF). Its concentration is elevated in depression and anorexia nervosa and decreased in Cushing's syndrome and Alzheimer's disease, and atypical depression. Interestingly, ACTH immunoreactive concentrations in the CSF follow the direction of CRH in the above conditions, suggesting that arcuate nucleus ACTH rather than pituitary ACTH is detected in the CSF.

We examined patients with atypical, seasonal depression. These patients develop dysthymia in the winter months, gain weight and are complaining of fatigue and somnolence. In these patients ovine CRH testing in the summer was totally normal, whereas in the winter it was indicative of secondary hypocortisolism. Interestingly, light therapy normalized the CRH response. We obtained similar data suggesting HPA axis hypofunction in patients with the chronic fatigue syndrome, a condition characterized primarily by fatigue, as well as in patients with fibromyalgia. These findings suggest that a spectrum of diseases exist, in which the pathophysiology is characterized by hypofunction of the central stress system. These syndromes contrast to melancholic depression, panic anxiety and anorexia nervosa, in which we have hyperactivity of the CRH system.

A state-independent abnormally functioning stress system (hyper- or hypo-functioning) might be either a result of different heredity or because an environmental stimulus was exerted at some critical period of life, usually during development (antenatally, infancy, childhood). A state-dependent abnormally functioning stress system, on the other hand, should revert completely to normal once the organism returns to a normal nonpathologic state. Unipolar melancholic depression or atypical depression have alterations of the HPA axis on opposite ends of a spectrum, which could be hereditary and/or constitutional (i.e. a trait) or state-dependent. Evidence from

experimental animals and humans accumulates, which indicates that the former is most likely the case. We examined several groups of patients and normals with the purpose of understanding better the relation between personality, stress, depression, and the HPA axis. These were sexually abused girls with dysthymia several years after the abuse took place, children of one or two parents with depression and adolescents with depression, as well as appropriate controls for each of the groups. All of these patients had standardized psychological testing, an oCRH test and measurement of 24h urinary free cortisol excretion. Sexually abused girls had a blunted ACTH response, a normal cortisol response and normal urinary free cortisol excretion. Our interpretation was that these patients had constitutional hypersecretion of CRH but normal negative feedback controls that allowed normalization of free cortisol. Children of one or two parents with depression had normal oCRH testing and urinary cortisol excretion. Adolescents with depression also had normal oCRH testing and urinary cortisol excretion. In the latter two groups we have the following potential explanations: (1) they have normal CRH secretion and the locus of depression is outside the stress system; (2) they are a mixed group of low and high CRH secretors and the averaging is normal; we saw no bimodal distribution; (3) they are low secretors primarily, and our means of examination failed to detect their abnormality; indeed, the age of onset for atypical depression is adolescence and for melancholic depression the third decade of life.

We have found that African Americans have higher plasma ACTH levels than Caucasians in response to CRH. Further study demonstrated that this is due to different metabolism of ACTH 1-39 in blacks vs. whites, with identical ACTH bioactivity, however, in both races.

We have cloned and sequenced the 5' regulatory region of the human CRH gene and identified recognizable signal sequences. The functions of some of these sequences were studied. This gene has 2 different promoters with potentially different regulatory sensitivities, and, in addition, 4 half perfect estrogen-responsive elements that respond positively to estradiol in an estrogen receptor-mediated fashion. The latter suggests that the sexual dimorphism of psychiatric diseases characterized by altered CRH secretion in humans, such as depression and anorexia nervosa, might be explained by the differences in the regulation of this gene.

We hypothesized that marmosets of the species Callithrix jacchus might represent a potentially interesting model for human depression. These New World primates maintain a monogamous breeding system with the males and older siblings participating in the rearing of infants. Subordinate males or females or offspring in the family are reproductively suppressed. Disruption of societal bonds, such as separation of the infant from its parents leads to significant behavioral and physical changes. These include in adults, withdrawn behavior, decreased feeding, loss of weight, and death ("wasting syndrome") and in infants delay or complete cessation of growth and abnormal behaviors. We have documented profound changes in hormones of the HPA axis with degree of subordination, and the wasting syndrome and have shown defective growth of infants abused by immature parents. The growth retarded infants catch up length-wise and improve behaviorally at later stages of their development.

B. Regulation of the Hypothalamic-pituitary-adrenal (HPA) Axis In Vivo and In Vitro.

It has become clear from our studies that opioids are involved in the regulation of the human hypothalamic-pituitary-adrenal axis. Morphine both suppresses the CRH-secreting neuron and/or stimulates some corticotroph suppressing factor. The overall effect of opioids is to suppress the hypothalamic-pituitary-adrenal axis. It has become known from other laboratories that catecholamines, vasopressin and oxytocin stimulate the corticotroph in vitro. Vasopressin also shows synergy with CRH in vivo when the peptides are administered together.

The possibility of peripheral extrahypothalamic CRFs or CRH CRFs participating in the regulation of the HPA axis and playing, in addition, other roles in homeostasis during stress, is a lead that we have followed. CRH at pharmacologic doses causes marked peripheral vasodilation in primates with a concomitant decrease in blood pressure. The major vessels involved were the mesenteric artery and to a lesser extent the iliac arteries. From a survey of peripheral tissues for CRH receptors we have found such receptors in the adrenal medulla and sympathetic ganglia. These receptors are coupled to cyclic AMP and to catecholamine and enkephalin secretion. Receptors on blood vessels or perivascular mast cells may also be present. We are actively searching for

peripheral extrahypothalamic (tissue) corticotropin-releasing or CRH releasing-factors. These factors appear to have different immunoreactivity from that of hypothalamic CRH. Immune system products, such as interleukins 1 and 6, thymosin and TNF- α , or tissue growth factors, such as epidermal growth factor, are potential candidates. We demonstrated that epidermal growth factor stimulated ACTH secretion in primates at doses or concentrations equimolar to those of CRH. We also examined the in vivo effects of TNF- α and platelet activating factor (PAF). Both substances produced a CRH-mediated response of the HPA axis of intact rats. We also examined recombinant interleukin-6 (IL-6) in humans. It appears to be the most potent ACTH secretagog known. In a dose- and time-dependent fashion, IL-6 stimulated CRH, and additionally, parvocellular AVP, magnocellular AVP, pituitary ACTH and adrenal cortisol secretion (see project Z01 HD 00615-15 DEB).

In the search of tissue CRF(s) we have examined two models of stress. First, patients undergoing neck exploration or major abdominal surgery and receiving identical perioperative sedation and anesthesia were examined before, during, and after surgery. In the former group we saw only very low plasma concentrations of CRH and mild activation of the HPA axis and the adrenomedullary system during surgery. ACTH and cortisol secretion was continuous rather than pulsatile and the ratio of ACTH to cortisol decreased when compared to basal secretion. These results suggest both the possible presence of a long-acting tissue CRF and of an adrenal component in surgical stress. The major elevation of cortisol, ACTH and epinephrine took place during anesthesia reversal. In contrast, in patients undergoing major surgery there was major activation of the HPA axis both during surgery and during recovery. In these patients, we found elevated concentrations of circulating CRH and profound elevations of AVP, ACTH, cortisol, catecholamines and neuropeptide Y, a peptide which is co-secreted with catecholamines and which has ACTH-like properties. Interestingly, secretion of CRH and ACTH was pulsatile with relative concordance of the pulses which took place approximately every 30-35 min.

Second, we examined the stress of dynamic exercise using a treadmill. Dynamic exercise is a primal component of the "fight or flight" response. The HPA axis as well as vasopressin, growth hormone, aldosterone, and prolactin secretion, are activated during exercise in an exercise intensity-dependent fashion correlating well with both percent of maximal O₂ consumption (VO₂max) and plasma lactate elevations. Trained subjects could perform high workloads with minimal activation of the axis, a result of chronic adaptation. There was a clearcut relationship between the exercising muscle and the HPA axis.

Upon CRH stimulation highly trained subjects (obligate athletes) had obvious evidence of hypercortisolism (blunted ACTH and cortisol responses and high baseline cortisol) suggesting chronic alterations of their HPA axis reminiscent of those seen in melancholic depression, anorexia nervosa, and chronic active alcoholism. We pursued this lead by psychometric testing of highly trained runners. It appears that during sustained physical training the affect of athletes is normal, suggesting that the self-imposed obligatory exercise in these athletes may be correcting some inherent mood disorder.

To extricate potential differences in the activity of the stress system within the normal population we performed a 90 VO₂max exercise test 2h after administration of a high dose of dexamethasone (4 mg). Two populations of subjects were distinguished. Approximately two thirds of the subjects were completely suppressed, while one-third had marked ACTH, AVP, and cortisol responses, which broke through the suppression of the glucocorticoid. The latter group also scored higher than the former group in the Spielberger anxiety scale. All volunteers had a normal single dose dexamethasone suppression test. The question arises as to whether the latter group is carrying a hereditary and/or constitutional trait of stress system hyperactivity and, hence, increase vulnerability to melancholic depression and anxiety disorders. Could the dexamethasone - exercise test be superior to dexamethasone or oCRH tests in revealing stress system hyperactivity? Could modifications of this combined test also help revealing a hypoactivity group, whose members would be vulnerable to develop atypical depression, chronic fatigue/fibromyalgia syndromes or autoimmune disorders (see project Z01 HD 00615-15 DEB)?

We have developed a hypothalamic organ culture system in which we study the in vitro regulation of hypothalamic CRH secretion directly. We have shown that serotonin, acetylcholine and the catecholamines (alpha receptors) stimulate CRH secretion whereas the GABA/benzodiazepine system is inhibitory. Glucocorticoids,

ACTH and β -endorphin, and CRH exert negative feedback (long, and short, and ultra short feedback loops, respectively). Alpha-MSH, and CLIP have suppressing effects on the CRH neuron. Interestingly, epidermal growth factor, interleukin 1, tumor necrosis factor- α , interleukin 2, interferon- γ , platelet activating factor (PAF) and several eicosanoids show strong CRH-releasing properties. The responses to interleukin 1 and TNF- α could be inhibited by prostaglandin synthesis blockade. These substances may represent some of the putative links between the immune-inflammatory response and the endocrine system. Recently, we demonstrated that vasopressin is a potent stimulus of CRH secretion, an effect that could be antagonized with vasopressin receptor antagonists of the V_3 (V_{1B}) type. This is interesting in light of the co-presence of CRH and vasopressin in the paraventricular nucleus and the known synergistic effects of CRH and vasopressin on ACTH secretion.

We have examined the effects of hypothyroidism and hyperthyroidism on the activity of the HPA axis and in particular the CRH neuron. We have found that they are, respectively, associated with hyposecretion and hypersecretion of CRH, a phenomenon that could explain the depressive symptomatology seen in both states. Using similar methodology, we have shown that cholecystokinin stimulates CRH secretion via peripheral vagal receptors and that neuropeptide Y has stimulatory effects upon the HPA axis at two levels: the hypothalamic CRH neuron and the adrenal cortex itself.

Pregnancy is the only known physiologic state in man in which CRH circulates in plasma at levels expected to cause activation of the pituitary adrenal axis (100 pg/ml up to 4000 pg/ml). These levels gradually increase even further during labor. Plasma CRH concentrations return to undetectable (< 15 pg/ml) following delivery. Interestingly, it has been known for years that the last 2-3 months of pregnancy are characterized by hypercortisolism at a degree similar to what has been observed in severe depression and anorexia nervosa. We have developed an *in vitro* perfusion system in which full thickness placenta fragments are kept in culture. These fragments contain a 1300 nucleotide long mRNA which hybridizes with a CRH-specific cDNA probe. Glucocorticoids, prostaglandins, catecholamines, oxytocin and vasopressin have no inhibitory or stimulatory effects on placental secretion of CRH in this model. The latter is chromatographically identical to hypothalamic CRH. Our perfused placenta fragments also secrete immunoreactive beta-endorphin, α -MSH, and A-dynorphin, chromatographically identical to synthetic human beta-endorphin, α -MSH, and A-dynorphin, respectively. Both CRH and oxytocin stimulate markedly the secretion of beta-endorphin, α -MSH and A-dynorphin by the placenta. These findings have raised the following questions: 1) Does the high intra-pregnancy CRH suppress hypothalamic secretion of CRH (via cortisol negative feedback) and therefore lead to a brief postpartum adrenal insufficiency state, followed by a later rebound of endogenous hypothalamic CRH secretion 6-8 weeks later? Interestingly, the very common "postpartum blues", occur in the first week postpartum whereas postpartum depression occurs 1-3 months later. 2) What regulates the secretion of placental CRH? Mechanical contraction or ischemia of the placental may be responsible for the elevations of plasma CRH seen during labor. 3) What is the function of pregnancy plasma CRH other than stimulating the maternal pituitary-adrenal axis? Do the vasodilatory properties of CRH play a role in labor? For instance, CRH-induced superior mesenteric vessel dilation may protect the maternal intestinal tract from ischemia. Moreover, circulating CRH might regulate uterine blood vessel flow towards the placenta. The latter was recently shown convincingly by an Australian group.

We recently completed a study examining the circadian and pulsatile activity of the HPA axis in normal pregnant women in the third trimester of pregnancy (32 wks, before the concentrations of CRH-BP in plasma decline). We found the placental CRH is secreted in a pulsatile but noncircadian fashion. Crosscorrelation demonstrated a positive correlation between placental CRH and ACTH and cortisol, however the concurrent presence of the circadian rhythm of ACTH and cortisol suggests that the circadian rhythmicity of ACTH and cortisol are maintained by the other major ACTH secretagogue, parvocellular AVP, which is also secreted in a pulsatile and circadian fashion, as parvocellular CRH. Naturally, levels of AVP in the systemic circulation were extremely low, as a result of peripheral proteolysis characteristic of the pregnant state.

We recently completed the analysis of a prospective, longitudinal study of pregnant-postpartum women which included psychometric testing and CRH testing in the postpartum. Overall, all women were euthymic while pregnant and had a transiently suppressed CRH neuron in the postpartum. When the group that developed

blues/depression was compared to the women that remained euthymic, there was a clear difference between the two groups with the former's suppression being more profound and longstanding than the latter's. These findings suggest that the postpartum blues/depression syndromes should be included in the stress system hypofunction states. If women during the postpartum had a defective HPA axis response to inflammatory stimuli, such as IL-6, this would provide an explanation for their increased vulnerability to autoimmune/inflammatory disease during this period of their life (see project Z01 HD 00615-15 DEB).

C. Roles and Actions and Pathophysiology of HPA and HPG Axes Hormones

Glucocorticoids - Glucocorticoid Resistance Syndromes:

Glucocorticoids have multiple actions which are essential in maintenance of homeostasis in both the resting (unstressed) state and during stress. The effects of glucocorticoids that are important on maintenance of resting homeostasis have been called "permissive or normalizing".

Glucocorticoids act upon their target tissues by modulating gene expression. This is attained by the interaction of a glucocorticoid-glucocorticoid receptor complex with specific sites in the nucleus, that regulate the expression of genes coding for function-specific proteins. We had the opportunity to study several large kindreds and individuals with generalized glucocorticoid resistance that had alterations of their glucocorticoid receptors, several New World primate species with generalized glucocorticoid and other steroid hormone resistance, and recently, New World prairie voles with generalized glucocorticoid resistance. We have obtained permanent cell lines from such individuals and species and are studying them in an attempt to understand the molecular mechanisms of hormone resistance and using them as an experiment of Nature to understand fundamental principles of glucocorticoid action. New World primates in addition to glucocorticoid resistance have progesterone, estrogen, aldosterone, androgen and vitamin D resistance. We demonstrated abnormalities in their glucocorticoid, progestin, estrogen and mineralocorticoid receptor. The animal model is of particular interest because its generalized steroid hormone resistance suggests a common link among the six classes of steroid hormones as they interact with their receptor and "acceptor" sites to modulate gene expression.

We have cloned, sequenced and performed *in vitro* mutagenesis studies with the glucocorticoid receptor of the patients and nonhuman primates. We found two different single amino acid substitutions in the steroid binding domain of the glucocorticoid receptor, which were responsible for the decreased affinity and effect of the glucocorticoid receptor in two different probands, that of the initial kindred and a subsequent interesting sporadic case. Homozygosity for the defect in the first kindred led to severe disease, whereas heterozygosity was associated with mild hypercortisolism, without overt clinical manifestations. The defect in our sporadic case with glucocorticoid resistance was a novel, *de novo* nonconservative amino acid substitution in the hinge region the glucocorticoid receptor, which not only abolished all agonist activity of the mutant glucocorticoid receptor, but also caused it to be a potent negative inhibitor of the normal receptor produced by the unaffected allele. The patient had moderate glucocorticoid resistance with hypertension and infertility. In his mid 30's, this patient developed also pituitary Cushing's disease. His corticotropinoma expressed P53 activity, suggesting that decreased glucocorticoid feedback due to glucocorticoid resistance of the corticotroph and of the hypothalamic neurons producing its secretagogues may be an early step in pituitary tumorigenesis. We have several additional families with glucocorticoid resistance whose glucocorticoid receptor we are evaluating now in great detail. Thus, a splice site micro deletion was found, resulting in expression of only one of the alleles, and associated with 50 percent reduction in receptor number and hyperandrogenism in the proband of this kindred and biochemical hypercortisolism in the affected brothers and father. Our results indicate that glucocorticoid resistance results from multiple defects and can be autosomal recessive, with heterozygotes expressing a subclinical phenotype, or autosomal dominant, with heterozygotes presenting with disease. We also have 2 families in whom we found no structural abnormality of the glucocorticoid receptor gene. We consider alternative mechanisms.

We obtained additional evidence that glucocorticoid resistance could lead to corticotropinoma formation. One of 4 patients with Nelson's syndrome was found to have a somatic nonsense mutation resulting in a frame-shift

and a truncated, inactive glucocorticoid receptor. Interestingly, none of the 4 tumors expressed abnormal P53 activity.

Glucocorticoids- An Endogenous Determinant of Glucocorticoid Sensitivity:

The human glucocorticoid receptor gene, located in the long arm of chromosome 5 consists of 10 exons. Exons 9 α and 9 β can be alternatively spliced into the mRNA to encode for the classic glucocorticoid receptor α and a nonligand-binding receptor which differs only in the C terminus. We demonstrated that mRNA of the β isoform is expressed in all human tissues examined and that in a co-transfection system in vitro it is able to antagonize the effects of glucocorticoids mediated by the α isoform. We concluded that the ratio of GR α to GR β may determine the sensitivity of a tissue to glucocorticoids.

Glucocorticoids - Effects on Growth:

We examined the influence of hypercortisolism on basal and stimulated growth hormone (GH) secretion. Young patients with Cushing's syndrome have profoundly suppressed spontaneous and provoked GH secretion. This deficiency takes over a year to correct, suggesting a rather protracted direct or indirect effect of the hypercortisolism on the somatotroph. Interestingly, during the convalescence year, levels of insulin-like growth factor I (IGF-I) in plasma remain normal, while those of IGF-binding protein 3 decrease, providing an explanation for the continuing growth of pediatric patients after cure of hypercortisolism, even though the levels of GH are suppressed. We found that patients who had Cushing's syndrome during their growth years lost on the average 7 cm of their final height, when they were followed up as adults.

Glucocorticoids - HPA Axis Feedback Effects via Type I Receptors:

The glucocorticoid receptor type I (mineralocorticoid receptor) is profoundly involved in the feedback regulation of the HPA axis in the rat. We demonstrated that the mRNA of this peptide is also expressed in Old and New World primates, in similar regions of the CNS. We examined its importance by administering specific type I antagonists to normal humans but found no effect of such antagonists on basal and CRH-stimulated cortisol secretion.

Sleep and δ -Sleep-Inducing Peptide in Cushing's Syndrome:

Patients with Cushing syndrome suffer from sleep disorders. We have developed an assay for δ -sleep-inducing peptide to study these patients in synchrony with continuous EEG monitoring. Preliminary study demonstrates that although δ -sleep induced peptide immunoreactivity is decreased in Cushing syndrome, its relationship with the sleep disturbances observed in this condition is tenuous.

Aldosterone Resistance-Pseudohypoaldosteronism:

Pseudohypoaldosteronism is a congenital condition characterized by profound salt loss, hypokalemia and acidosis in the presence of extremely elevated levels of plasma and urinary aldosterone. The resistance to aldosterone can be limited to the kidney or generalized to all mineralocorticoid target tissues. The clinical severity of the syndrome improves with age. About 2/3 of the cases are sporadic, while 1/2 are familial. We studied several sporadic cases for the presence of defects in the mineralocorticoid receptor to find no pathogenetic mutations. We have focused our efforts on a postreceptor mechanism, hopefully to find mutations in one of the 4 subunits of the amiloride-sensitive sodium channel.

ACTH Resistance - Hereditary Isolated Glucocorticoid Deficiency:

ACTH regulates glucocorticoid secretion by the adrenal cortex via its recently cloned receptor. This membrane protein belongs to the superfamily of G protein-coupled receptors, forming its own distinct class with the receptors for α MSH. We mapped the gene of the ACTH receptor in the short arm of chromosome 18 and studied families with hereditary isolated glucocorticoid deficiency (congenital insensitivity to ACTH) for potential defects of this gene. We found that point mutations resulting in premature termination of the receptor or nonconservative amino acid substitutions in key functional areas of the molecule cause the disease. Heterozygote carriers are clinically healthy but revealed by CRH testing, since they have markedly exaggerated responses of ACTH to this hormone.

Testicular LH Resistance - Leydig Cell Hypoplasia - Ovarian LH Resistance:

LH regulates testosterone secretion by Leydig cells and ovulation and corpus luteum formation by the ovarian follicle via its recently cloned receptor. This membrane protein belongs to the superfamily of G protein coupled receptors, forming its own distinct class with the receptors for the other glycoprotein hormones. This group studied families with hereditary primary Leydig cell failure with genetic male members that presented with phenotypically female external genitalia or micropenis and genetic females with primary amenorrhea and infertility. They found that point mutations resulting in premature termination of the receptor or nonconservative amino acid substitutions in key functional areas of the receptor molecule caused the disease. Heterozygotes were clinically healthy. Genetic females with complete absence of the LH receptor developed normally in utero and had a normal pubertal development, but remained anovulatory.

D. Mechanisms of Adrenocortical Tumorigenesis

We have studied plausible mechanisms of adrenocortical tumorigenesis with interesting preliminary results. All tumors are monoclonal. About one third of malignant adrenocortical tumors and both available adrenocortical cancer cell lines have abnormalities of the p53 gene, while none, including the two cell lines and a series of adenomas has activating mutations of the ACTH receptor gene or mutations of the G proteins.

In our patient population, we had 5 kindreds and several sporadic cases with primary pigmented nodular adrenocortical disease (PPNAD) or micronodular adrenal disease, a major component of Carney complex, a multiple neoplasia/lentiginosis syndrome transmitted in an autosomal dominant fashion. In collaboration with J.A. Carney of the Mayo Clinic, we included an additional 6 kindreds in our series and attempted chromosomal mapping of the syndrome by linkage analysis. We indeed mapped the defective gene of the complex within a ~6 cM region of the short arm of chromosome 2 in band p16. A small number of genes are known in this area, such as those of calcineurin B and two DNA repair enzymes, previously shown to be involved in nonpolyposis colon cancer and/or microsatellite instability.

Significance to Biomedical Research and the Program of the Institute:A. Clinical Applications and Implications of CRH

Different patterns of plasma ACTH and cortisol responses to CRH have been noted in different diseases, including hypercortisolism and hypocortisolism. The CRH stimulation test appears to differentiate between Cushing's disease and the ectopic ACTH syndrome. In addition, it appears to be of diagnostic value in distinguishing between Cushing's disease and hypercortisolism due to depression. Because of its biological properties hCRH is an important means to study the normal physiology of the hypothalamic-pituitary-adrenal axis. In psychiatric diseases characterized by hypercortisolism, such as melancholic depression and anorexia nervosa, a pathophysiologic role has been suggested for CRH and this hypothesis is currently tested. Were it true, new treatments could be developed with major goal to prevent CRH hypersecretion or action. We plan to examine

the efficacy of a newly discovered nonpeptide CRH antagonist, which could be used orally and which is expected to cross the blood brain barrier.

Our recent studies with atypical depression, the chronic fatigue/fibromyalgia syndromes and the postpartum blues/depression syndromes suggest that a spectrum of human pathologic conditions exist in which the central CRH system is hypofunctional. Activation of the CRH system in such patients may help with the resolution of the clinical picture. Also, CRH agonists or molecules that displace CRH from its binding protein might be useful in the treatment of these conditions. We study the psychological, social and cognitive changes in patients with Cushing's syndrome before and after cure of their hypercortisolism. It is of interest that we have found similarities between Cushing's syndrome both before and after cure and atypical depression, probably stemming from the same pathologic decrease in CRH secretion. We found that the common marmoset could be an excellent model of human depression and psychosocial retardation. We found that sexual abuse in childhood has profound prolonged effects on the hypothalamic-pituitary-adrenal axis, as well as on the affect of these individuals.

B. Regulation of the Hypothalamic-Pituitary-Adrenal Axis In Vivo and In Vitro

We determined the changes of the HPA axis during the stress of surgery or exercise. We have also demonstrated that hyperthyroidism and hypothyroidism are respectively, associated with hypersecretion and hyposecretion of CRH which might explain the presence of depressive symptomatology in both conditions. Several peripheral substances secreted during stress (interleukin 1 and 6, TNF- α , epidermal growth factor, platelet activating factor, prostanoids) appear to be CRH secretagogues of physiologic relevance. We determined the major stimulatory (serotonin, acetylcholine, norepinephrine) and inhibitory (GABA/benzodiazepine system) neurotransmitters of hypothalamic CRH secretion *in vitro* and demonstrated the presence of a long, a short and an ultra-short feedback loop respectively with glucocorticoids, ACTH and β -endorphin, and CRH, all playing a role at the level of the CRH neuron. These advances from basic science are helpful in understanding the various disorders of the HPA axis that we study. We have demonstrated that the human placenta secretes large quantities of CRH, POMC-derived peptides (primarily δ endorphin and α -MSH) and dynorphin A and that CRH exerts autocrine and/or paracrine actions on the placenta by causing δ -endorphin, α -MSH and dynorphin A secretion by this tissue. These results may influence our way of thinking about the physiology and pathophysiology of pregnancy and labor and delivery. We confirmed our hypothesis that the postpartum blues/depression represent a state of "adrenal suppression" characterized by suppression of hypothalamic CRH secretion and symptoms of atypical depression. We have examined in great detail the regulatory region of the human CRH gene.

C. Roles, Actions, and Pathophysiology of HPA and HPG Axis Hormones

Pathologic mutations and/or deletions of the glucocorticoid receptor gene are responsible for human familial glucocorticoid resistance. Generalized or tissue-specific resistance can lead to human tumorigenesis. The ratio of the two isoforms of the glucocorticoid receptor may define a tissue's sensitivity or resistance to glucocorticoids. The glucocorticoid receptor isoforms may participate in the translocation of AIDS virus proteins, such as vpr, which by activating the glucocorticoid receptor may stimulate viral replication via the GREs present in the promoter region of HIV. Also, by exerting glucocorticoid effects, the glucocorticoid receptor - vpr complex may cause further immunosuppression. Thus, the glucocorticoid receptor may participate in the hallmark manifestations of AIDS, may hasten the conversion from the carrier to the disease state and may curtail the terminal phase of the disease.

A transcription or other factor common to all steroid hormones may be defective in New World primates. We suggest that the glucocorticoid receptor may be involved in highly prevalent human diseases, such as obesity, hypertension, depressive/anxiety syndromes and autoimmune diseases by providing excessive or defective glucocorticoid activity to a tissue.

It is important to pursue our search for the molecular defect of pseudohypoaldosteronism. We have defined common human mineralocorticoid receptor and amiloride-sensitive sodium channel subunit α polymorphisms

which may be useful in the pursuit of the genetic determinants of hypertension.

We have examined several families with hereditary isolated glucocorticoid deficiency (ACTH resistance) for possible defects of the ACTH receptor gene. We found pathologic mutations, altering the receptor and causing the disease. The heterozygote parents and grandparents have abnormal ACTH responses to ovine corticotropin releasing hormone, suggesting that this test can be employed for the ascertainment of the carrier state in this disorder. We found that the triple A syndrome, which has hereditary isolated glucocorticoid deficiency as a component (Adrenal insufficiency, Achalasia, Alacrima), is not associated with molecular defects of the ACTH receptor.

We have defined the LH receptor gene as a locus of the defect in complete male pseudohermaphroditism (Leydig cell hypoplasia) and micropenis in genetic males and in anovulation and primary amenorrhea in genetic females.

D. Mechanisms of Adrenocortical Tumorigenesis

Adrenal cancer continues to represent a rare but aggressive carcinoma that affects children and young individuals and responds little in terms of medical therapy. Every effort should be placed in understanding this disease, in achieving early diagnosis and in devising effective cures.

Our Carney complex research may not only elucidate the molecular pathophysiology of a rare multiple neoplasia/lentiginosis syndrome that afflicts children and young individuals but also may help unravel important physiological processes and mechanisms of tumorigenesis, as it has happened already with other rare syndromes.

Proposed Course:

A. Clinical Applications and Implications of CRH

1. Is CRH Involved in the Pathophysiology of Affective Disorder?

a) We have formulated the general hypothesis that CRH plays a pathophysiologic role in psychiatric disease characterized by hypersecretion or hyposecretion of this peptide. CRH antagonists or inhibitors of CRH secretion as well as CRH agonists or stimulants of CRH secretion, will be used as tools to test this hypothesis. Animal models for depression would be ideal to begin such testing.

b) It is evident from studies by S. Levine and S. Suomi that neonatal separation of rats or monkeys from their mothers can be associated with hyperactivity of the hypothalamic-pituitary-adrenal axis and behavioral disturbances throughout their life. We obtained similar results in sexually abused girls long after the abuse took place. These findings suggest that there are critical periods in human life as well, associated with permanent alterations in the reactivity to stress. The hypothesis that CRH is involved in this early sensitization can be examined in various ways. We plan to use CRH antagonists to reverse the clinical picture of animals that develop the syndrome.

2. Can We Unravel Affective Disorder Traits?

If for genetic or constitutional reasons the number of hypothalamic CRH neurons in patients prone to develop melancholic depression/anxiety disorders were increased, one would hope that a strong stimulus of the CRH neuron, such as IL-6, would be able to distinguish this group from normal controls. The same may be true for our dexamethasone-exercise test paradigm. Similarly, if for the same reasons the number of hypothalamic CRH neurons in patients prone to develop atypical depression, the chronic fatigue/fibromyalgia syndrome or autoimmune disease, were decreased, IL-6 might also be able to detect them.

3. Differential Diagnosis of Cushing's Disease from Pseudo-Cushing States

In spite of the assistance of the combined dexamethasone suppression/oCRH stimulation test, it is still difficult to differentiate some patients with depression and hypercortisolism from patients with mild Cushing's disease. Based on previously obtained information from human and in vivo and in vitro studies, we have designed a study which may assist us further with this differential.

Thus, the cytokine IL-6 administered acutely should stimulate the HPA axis of patients with pseudoCushing's (depression, chronic active alcoholism) but not that of patients with Cushing's syndrome of any etiology since in all instances hypothalamic CRH should be suppressed.

4. Postpartum "Blues" and Postpartum Depression

Following delivery of the fetus and the placenta, the mother loses a source of peripheral CRH production. This is likely to be associated with subphysiological cortisol secretion during the first days of the postpartum period, a result of a suppressed hypothalamic CRH neuron. Interestingly, 40-50% of postpartum women develop the postpartum blues during the first 2-3 weeks postpartum. We hypothesized that this syndrome was equivalent to glucocorticoid withdrawal, in a sense being an analogue of the steroid withdrawal syndrome of the post transsphenoidal surgery patient with Cushing's disease, who despite glucocorticoid replacement develops "postoperative blues".

Three weeks to three months postpartum, approximately 10% of women develop a frank form of depression which interferes with their overall function. Interestingly, this is the period of the putative hypothalamic CRH recovery. Our prospective study showed the alterations of the hypothalamic-pituitary-adrenal axis in the postpartum and the suppression observed was related to mood changes in the mothers. We anticipate that high levels of plasma CRH or high urinary free cortisol excretion pre-partum might predict the severity of the postpartum blues or depression syndrome. A larger study needs to be performed now to test this hypothesis. In a small subgroup, IL-6 should be administered, as discussed in project Z01 HD 00615-15 DEB, to test the hypothesis of diminished responsiveness to inflammatory stimuli in this period, which is characterized by increased vulnerability to autoimmune disease.

Growth and development of infants of postpartum depressed mothers might be affected to a degree proportional to the severity of the maternal syndrome. Interestingly, maternal circulating CRH and cord blood CRH concentrations are positively correlated. Thus, the infants should be followed as well.

B. Regulation of the Hypothalamic-Pituitary-Adrenal Axis In Vivo and In Vitro

1. Regulation of the Circadian Rhythmicity and Stress Activation of the Human and Rat Hypothalamic-Pituitary-Adrenal Axis

We have shown that continuous i.v. infusion of pharmacologic amounts of oCRH in normal volunteers leads to mild hypercortisolism (UFC 150-200 mcg/d) and preservation of the plasma ACTH and cortisol circadian rhythm. Interestingly, the secretory episodes of ACTH and cortisol continue to be seen despite the maximal circulating concentration, of CRH. On the other hand, CRH concentrations in the CSF have a circadian rhythm which is in opposite phase to the circadian rhythm of plasma cortisol concentrations. We have postulated that ACTH secretagogues other than CRH may be involved in the regulation of episodic secretion of ACTH and cortisol and the circadian rhythmicity of the HPA axis. Vasopressin is a such potential factor. We have available several (V_3) receptor vasopressin antagonists which will be administered continuously i.v. to test the hypothesis that vasopressin participates in the generation of the circadian rhythm. We have also generated antibodies against vasopressin and CRH with which we perform immunoneutralization studies in experimental animals.

The V_3 receptor antagonists could be employed to define the degree of vasopressin participation in the stress-related activation of the pituitary-adrenal axis in man. Thus, graded levels of exercise, or an insulin tolerance test, could take place while a vasopressin antagonist infusion is being administered.

2. Regulation of Placental CRH, POMC-Derived Peptides and Dynorphin A In Vivo and In Vitro

The possible association of placental CRH secretory episodes with uterine contractions will be examined by frequent, every 3 min serial sampling over 3 hours in end-stage labor.

Prostanoids and platelet activating factor will be examined as stimulants of placental CRH or POMC-derived peptides in vitro. Of particular interest are also several agents, including serotonin, epidermal growth factor (EGF), platelet derived growth factor (PDGF), interleukins 1 and 6, and tumor necrosis factor (cachexin), which cause hypothalamic CRH secretion in vitro. PDGF was recently found to mimic the effect of serum from preeclamptic women in causing lysis of cultured endothelial cells. It is of interest that preeclampsia is associated with elevated circulating CRH concentrations in pregnancy.

C. Roles, Actions and Pathophysiology of HPA and HPG Axes Hormones

1. Cloning of the Glucocorticoid Receptor Type II Gene in Patients with Glucocorticoid Resistance and in Animal Models

We are now examining the glucocorticoid receptor of members of several new families with glucocorticoid resistance. Also, we are pursuing several hypotheses for the mechanism(s) of glucocorticoid resistance in the 2 families in which no structural abnormalities of the glucocorticoid receptor gene were found. The aldosterone, progesterone, and estrogen receptor of the New World primates will be cloned also, with the assistance of probes obtained from the human. We postulate that the common link between all steroid hormones that is responsible for the generalized steroid hormone resistance in New World primates represents a common modulator of all steroid receptors. This could be an enzyme, such as a steroid receptor phosphokinase, a common trans-activating factor necessary for the interaction of the steroid receptor in the nucleus with their responsive elements, or a common steroid-class specific binding protein. We are characterizing the prairie vole as a glucocorticoid resistant animal and examine the molecular mechanisms of resistance in this species, as well.

2. Continuing Studies with the β -Isoform of the Glucocorticoid Receptor

There are many important questions remaining in this area. We need to demonstrate and quantify the actual protein content of each isoform in human tissues. We need to demonstrate the actual location of the β isoform within the cell and its relation to heat shock proteins. We need to examine the interaction of the β isoform not only with GREs, but also with transcription factors known to interact with glucocorticoid receptors and known to be influenced by this interaction. These include the cjun-cfos and the NF-kB/Rel A heterodimers. One could include here the HIV protein vpr, which apparently binds to the glucocorticoid receptor and translocates with it into the nucleus to potentially enhance viral replication and suppress host immune functions via classic glucocorticoid receptor-mediated pathways. This hypothesis is testable and we plan to pursue it. We will pursue also the potential involvement of GR β in human pathophysiology. Thus, we will examine tissues of patients with glucocorticoid-resistant asthma and other conditions for overexpression of the β -isoform, and tissues of patients with glucocorticoid hypersensitivity (metabolic syndrome X) for underexpression of the β -isoform.

3. Molecular Mechanisms of Aldosterone Resistance

We are pursuing a likely postreceptor site, the amiloride-sensitive sodium channel, with its 4 known subunits.

4. ACTH Receptor Studies in Patients with ACTH Resistance

We are now examining the ACTH receptor in several additional kindreds with isolated hereditary ACTH resistance, as well as kindreds with the triple A syndrome (Adrenal insufficiency, Achalasia, Alacrima). In the latter we will first attempt genetic linkage studies, once we have collected the necessary number of subjects.

5. LH Receptor Studies in Patients with LH Resistance

We are examining other potential candidates with defects of the LH receptor.

D. Mechanisms of Adrenocortical Tumorigenesis

We continue to examine major candidate genes potentially participating in adrenocortical tumorigenesis. These include those of p16, steroidogenic factor 1 and DAX 1.

We continue our two-prong approach towards elucidating the molecular pathophysiology of Carney complex. Thus, we examine candidate genes within the 2p16 region, in which we localized the gene and continue to cone down on a progressively smaller region, until we hopefully identify the actual gene.

Protocols:

Human

82-CH-45	Chrousos	Dose-Response Relationship for Single Doses of Corticotropin-Releasing Hormone (CRH) in Normal Volunteers and in Patients with Adrenal Insufficiency (active)
		This blanket protocol enables us to administer ovine corticotropin-releasing hormone (CRH) as a provocative test of the hypothalamic-pituitary-adrenal (HPA) axis in men, women, and children with potential alterations of this axis. The peptide continues to be an investigational drug (IND# 19802) and all data concerning its administration are captured and reported annually to the FDA. During the past year oCRH was administered under this and other approved protocols.
82-CH-125	Gold	Studies of Corticotropin Releasing Factors (CRF) and Related Neurotransmitters, Neurotransmitter Metabolites and Peptides in the Spinal Fluid of Patients with Cushing's Disease (completed)
86-CH-172	Laue	Dose Response Relationships between Exogenous ACTH and Adrenal Cortisol Secretion in Normal Volunteers and Patients with Abnormalities of the Hypothalamic-Pituitary-Adrenal Axis (completed)
88-CH-120	Chrousos	The Hypothalamic-Pituitary-Adrenal Axis in Pregnancy, the Postpartum Period, and in Postpartum Depression Syndromes (completed)

88-CH-115	Chrousos	Placental Secretion of Corticotropin Releasing Hormone during Pregnancy and Labor and Delivery (partially completed)
90-CH-125	Chrousos	Psychological, Social and Cognitive Changes in Children, Adolescents and Adults with Cushing syndrome (completed)
90-CH-124	Friedman	Circulating Hormones and EEG Monitoring in Patients with Cushing's Syndrome (completed)
90-CH-194	Yanovski	Inferior Petrosal Sinus Sampling for the Determination of ACTH Concentration in Normal Volunteers and in Patients with Disorders of the Hypothalamic-Pituitary-Adrenal Axis (active) This protocol allows a procedure which is diagnostic in Cushing's syndrome to be performed in normal volunteers and patients with abnormalities of the HPA axis, whom it would not benefit. This protocol, thus, provides valuable normal control samples and samples that may help us understand better the pathophysiology of the HPA axis.
91-CH-166	Chrousos	Hypothalamic-Pituitary-Adrenal Axis Function in Offspring of Affectively Ill Mothers vs Offspring of Normal Control Mothers (completed)
92-CH-181	Peeke	Energy Metabolism in Patients with Seasonal Affective Disorder (completed)
94-CH-134	Papanicolaou	Dose-Response Relationships for Single Doses of Recombinant Interleukin-6 in Normal Volunteers and Patients with Disorders of the Hypothalamic-Pituitary-Adrenal Axis (active) This protocol concerns the administration of recombinant interleukin-6 to normal volunteers and patients with disorders of the HPA axis. Interleukin-6 is an investigational drug (IND# 5419), with which we have already obtained some experience from a phase 1 study of cancer patients done by NCI. A dose-response curve in normal volunteers has been completed and a dose has been selected to be administered to the various patient groups listed in the protocol. The hypothesis of the protocol is that as a hypothalamic stimulus of CRH, acute administration of interleukin-6 will distinguish biochemically not only patients with a clearly disordered HPA axis, i.e. Cushing's <u>vs.</u> pseudoCushings's <u>vs.</u> melancholic depression <u>vs.</u> chronic active alcoholism or melancholic <u>vs.</u> atypical depression, but also premorbid states of vulnerability, i.e. individuals prone to melancholic depression or anxiety disorders or individuals prone to atypical depression, chronic fatigue/fibromyalgia, postpartum blues or depression, alcoholism or autoimmune disorders.
95-CH-59	Stratakis	Definition of the Genotype and Clinical Phenotype of Primary Pigmented Adrenocortical Disease and Its Associated Conditions (Carney Complex)

This protocol allows the examination and obtaining of samples from patients with Carney Complex and their families. Samples include blood for leukocytes and DNA and tumors for cytogenetic and DNA studies. The first phase of the protocol, to collect sufficient numbers of samples to perform the initial mapping of the defective gene by linkage analysis has been completed. More precise localization and testing of candidate genes and examination of tumors for clues continue.

Animal

88-011	rat	Chrousos	Effect of Thyroid Hormones on Hypothalamic-Pituitary-Adrenal Function (completed)
89-036	rat	Chrousos	Regulation of Hypothalamic CRH & Pituitary ACTH Release (active)
91-025	rat	Chrousos	Heat Shock Protein 90 (HSP 90) and Glucocorticoid Receptor Gene Expression In Vivo: Effects of Adrenalectomy and Glucocorticoid Administration (completed)
92-031	rat	Chrousos	The Effects of Acute and Chronic Stress on Hypothalamic-Pituitary-Adrenal Axis and Sympathetic Nervous System Function of the Zucker (fa/fa) Rat (completed)
93-037	rat	Cizza	Regulation of the Hypothalamic-Pituitary-Adrenal Axis at Basal Conditions and During Immobilization in the Aged Male and Female Fischer 344/N Rat (active)

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HD 00623-12

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adrenal Physiology, Pathophysiology, and Molecular Biology

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.:	G.B. Cutler	Head	SDE, DEB, NICHD
Others:	K.M. Barnes	Chemist (Tech)	SDE, DEB, NICHD
	G.P. Chrousos	Chief, SPE, DEB	SPE, DEB, NICHD
	L. Laue	Adjunct Scientist	SPE, DEB, NICHD
	L. Mercado-Asis	Visiting Associate	SDE, DEB, NICHD
	D. Merke	Clinical Associate	SDE, DEB, NICHD
	L. Nieman	Head	URM, DEB, NICHD
	K. Oerter-Klein	Special Volunteer	SDE, DEB, NICHD
	J. Yanovski	Clinical Associate	SDE, DEB, NICHD

COOPERATING UNITS (if any)

J. Doppman, Radiology, CC; E. Oldfield, Surgical Neurology Branch, NINDS

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Developmental Endocrinology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.4

PROFESSIONAL:

2.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

We seek to advance understanding of the mechanisms that cause adrenal androgen secretion by the fetal adrenal zone prenatally and by the definitive adrenal cortex during adrenarche, and to improve the diagnosis and treatment of disorders that cause excess adrenal androgen or glucocorticoid secretion, such as premature adrenarche, congenital adrenal hyperplasia, adrenal neoplasms, idiopathic hirsutism, polycystic ovary syndrome, and Cushing syndrome. We also seek to clarify the pathophysiology of primary adrenal insufficiency (Addison's disease) and secondary adrenal insufficiency and to improve the treatment of these conditions.

Children with congenital adrenal hyperplasia are being enrolled into a study to test the hypothesis that growth can be normalized, and the side effects of supraphysiologic glucocorticoid treatment avoided, by a regimen of antiandrogen, aromatase inhibitor, and reduced hydrocortisone dose. Additionally, the potential to cure the 21-hydroxylase deficiency form of congenital adrenal hyperplasia by gene therapy is being explored in a newly recognized animal model of this disorder.

Patients with Cushing syndrome or a pseudo-Cushing state are being studied by several new diagnostic methods to determine the relative diagnostic efficiency of these new methods compared to the old. Oncogene expression in corticotropinomas is also under study in an attempt to elucidate the molecular basis of Cushing disease.

Project DescriptionObjectives:

This project seeks to increase our understanding of adrenal physiology, pathophysiology, and molecular biology. The goals of the project are the study of fetal adrenal development, adrenarche, and disorders of adrenal function. The principal disorders under study are congenital adrenal hyperplasia, Cushing syndrome, and pseudo-Cushing states.

Methods Employed:

- 1) Fetal adrenal development - adrenal gland histology and steroid radioimmunoassay in newborn marmosets treated with substances that have been postulated to be trophic for the fetal adrenal zone.
- 2) Congenital adrenal hyperplasia - steroid radioimmunoassay, evaluation of new treatments such as LHRH analog, antiandrogen (flutamide) and aromatase inhibitor (testolactone); adrenal transplantation and transgenic techniques, using a newly recognized mouse model of 21-hydroxylase deficiency, to test the hypothesis that the enzyme deficiency can be cured through these approaches.
- 3) Cushing syndrome and pseudo-Cushing states - evaluation of corticotropin-releasing hormone, overnight dexamethasone, overnight metyrapone, and combined dexamethasone/petrosal sinus catheterization as new approaches for the differential diagnosis of Cushing syndrome; oCRH test to predict recurrence of Cushing syndrome after successful microadenectomy; oncogene expression in corticotropinomas (extraction of tumor mRNA and identification and analysis of suspected oncogenes by RT-PCR).
- 4) Adrenal insufficiency - Steroid and peptide radioimmunoassay to study feedback regulation of adrenocorticotropin (ACTH) by cortisol.

Progress:1. Congenital adrenal hyperplasia

a. Glucocorticoid dose schedule in the treatment of CAH

Previous studies suggested that it might be possible to develop a once-daily hydrocortisone schedule for the treatment of CAH. To test this hypothesis, once-daily and twice-daily schedules were compared in a double-blind, randomized cross-over trial of 2 years' duration (one year on each arm). Total hydrocortisone dose was permitted to be adjusted throughout both arms, as would be the case in clinical practice, and thus the total daily hydrocortisone dosage for each arm was one outcome variable of the study. Despite a 40% increase of hydrocortisone during the once-daily regimen, both the clinical and biochemical measures of control of CAH deteriorated significantly compared to the twice-daily regimen. We concluded that CAH cannot be controlled as effectively with a once-daily regimen, even with a substantial increase in total daily hydrocortisone dose.

b. Supplemental salt administration in patients with CAH

Hypovolemia due to impaired mineralocorticoid synthesis is a major stimulus to ACTH secretion in CAH. Although administration of fludrocortisone should in theory correct mineralocorticoid deficiency, the appropriate dose of fludrocortisone depends upon dietary salt intake and varies accordingly. Thus, patients on a fixed dose of fludrocortisone may have too much mineralocorticoid on some days and too little on others, due to fluctuations in dietary salt. We therefore initiated a randomized double-blind clinical trial to test the hypothesis that supplemental salt administration will reduce day-to-day fluctuation in ACTH secretion, and thus improve control of androgen secretion, by eliminating fluctuations in the hypovolemic stimulus to ACTH release. The results to date have indicated improved adrenal suppression, as predicted. However, the magnitude of the effect in children who are receiving fludrocortisone appears too small to be of clinical importance.

c. Treatment of CAH with a new regimen of flutamide, testolactone, and reduced hydrocortisone dose

The successful use of antiandrogen in familial male precocious puberty, combined with inhibition of androgen-to-estrogen conversion, has led us to initiate a similar approach in congenital adrenal hyperplasia. The protocol employs flutamide as the antiandrogen and testolactone as the inhibitor of androgen to estrogen conversion. The rationale for this approach is that androgen excess is an inevitable outcome of physiologic hydrocortisone dosage in CAH. Blockade of excess androgen offers the prospect of normal growth, whereas the conventional approach of suppressing excess androgen through supraphysiologic hydrocortisone dosage does not, because of the growth-inhibiting actions of glucocorticoid.

Preliminary results of this new approach have recently been analyzed. Compared to conventional treatment, the regimen of flutamide, testolactone, and reduced hydrocortisone dose produced an increase in plasma 17-hydroxyprogesterone levels and a significant decline in urine cortisol, linear growth rate, weight velocity, and bone maturation. No important adverse effects were observed. We conclude that the regimen of flutamide, testolactone, and reduced hydrocortisone dose improves the short-term control of growth and bone maturation in children with congenital adrenal hyperplasia. Long-term studies are required to determine whether this approach can normalize these children's growth and development.

d. Testicular adrenal rest tissue in boys with congenital adrenal hyperplasia

One of the most troublesome complications of CAH in boys is the development of testicular adrenal rest tumors. This complication is thought to arise because of increased ACTH levels acting on adrenal rest cells within the testis, an occurrence which has been described in approximately 10% of normal subjects. Thus, those boys with CAH who have adrenal rest cells within the testis are presumed to be at risk for this complication, whereas those who do not have such cells present are presumed not to be at risk.

Although at present there is no way to determine which boys are at risk, it would be desirable to detect these lesions as early as possible so that attempts could be made to assure the optimal degree of ACTH suppression. To improve detection, Avila and his colleagues have performed a detailed analysis of the gray scale and color Doppler ultrasound features of the testicular adrenal rest tissues in 8 boys with this rare condition. Through earlier detection, we hope to develop improved methods to prevent the progression of this complication.

2. Cushing syndrome

a. Pathophysiology of glucocorticoid-induced growth suppression

Previous studies in our laboratory showed that glucocorticoid-induced growth suppression could be induced in a single proximal tibial epiphysis by the local infusion of dexamethasone. The mechanism of this local growth arrest did not involve suppression of mRNA for growth hormone receptor in the affected epiphysis. Recently, we have used this growth suppression model to examine the hypothesis that catch-up growth after glucocorticoid withdrawal results from a neuroendocrine mechanism. Contrary to this hypothesis, catch-up growth after termination of the dexamethasone infusion was observed only in the epiphysis that had received dexamethasone. No change in growth rate was observed in the control contralateral epiphysis, or in the ipsilateral distal tibial epiphysis. Thus, catch-up growth following glucocorticoid withdrawal was a local phenomenon that occurred only within the affected growth plate.

b. Differential diagnosis

Despite recent advances in transsphenoidal surgery for Cushing disease, the remission rate for all patients undergoing transsphenoidal surgery has remained well below 100 percent. The causes of surgical failure include errors of diagnosis, including the failure to distinguish an ectopic from a pituitary source of ACTH and the failure to distinguish Cushing disease from pseudo-Cushing states, and inability of the surgeon to locate pituitary microadenomas during surgery.

We have recently made the following contributions in the area of differential diagnosis:

- 1) New criteria have been developed for the single-dose, overnight metyrapone test. Based on a pilot study of this simplified test, the diagnostic efficiency is nearly identical to that of the 2-day standard metyrapone regimen that we described in the *Annals of Internal Medicine* in 1994.
- 2) A novel combined dexamethasone/inferior petrosal sinus sampling (IPSS) test appears to improve the discrimination between pituitary Cushing disease and ectopic ACTH syndrome in patients with mild or intermittent hypercortisolism and in patients who have had recent medical treatment for hypercortisolism. Moreover, the absolute level of the CRH-stimulated plasma ACTH in the petrosal sinuses appears to distinguish patients with a pseudo-Cushing state from patients with Cushing disease.
- 3) Contrary to a recent report from another medical center, cavernous sinus sampling did not lead to improved diagnosis compared to inferior petrosal sinus sampling.
- 4) We reported the largest series to date on the diagnostic evaluation of pediatric Cushing's syndrome, showing that the diagnostic criteria developed previously for adults are also applicable to children and adolescents with this disorder.

1) New diagnostic criteria for the single-dose overnight metyrapone test

A pilot study involving 63 patients has been conducted to determine whether this simplified test, requiring 12 hours rather than the 48 hours for the standard test, is as effective diagnostically as the standard test. Stimulation of 11-deoxycortisol > 225-fold, or a decrease of plasma cortisol during metyrapone of < 45%, yielded a specificity of 100% and a sensitivity of 68% for the diagnosis of Cushing disease. The sensitivity of 68% was nearly identical to that obtained with the standard test in the same patients (67%). We conclude that the single-dose test, which avoids the need for timed urine collections, has nearly identical diagnostic efficiency as the standard test. Moreover, combining the results from the two tests increased sensitivity to 84%, which was significantly greater than with either test alone ($p < 0.02$). This illustrates the clinical value of performing additional tests when a single test yields negative or equivocal results. We ultimately envision that this test could be combined with two other noninvasive, single-dose tests (8-mg overnight dexamethasone test and the ovine corticotropin-releasing hormone test) to permit accurate non-invasive diagnosis of Cushing disease in nearly all subjects.

2) Combined dexamethasone/inferior petrosal sinus sampling (DEX/IPSS) test

One of the pitfalls of the IPSS procedure to distinguish ectopic from pituitary sources of ACTH is the patient with an ectopic source of ACTH who has had recent adrenal blockade or who has mild or intermittent hypercortisolism. The normal pituitary corticotroph cells in such patients may be unsuppressed, giving rise to a petrosal sinus-to-peripheral ACTH gradient that mimics the gradient seen in Cushing disease. To overcome this problem, we hypothesized that the administration of dexamethasone (8 mg per day for 2 days) prior to IPSS would fully suppress normal, but not adenomatous, corticotroph cells, leading to improved diagnosis. Preliminary results indicate that this is indeed the case, and that the DEX/IPSS test will improve diagnostic accuracy in this setting.

3) Selective cavernous versus inferior petrosal sinus sampling for differential diagnosis of Cushing syndrome

To test the hypothesis that cavernous sinus sampling would generate higher central-to-peripheral ACTH gradients and obviate the need for concurrent CRH administration to localize occult pituitary microadenomas in Cushing disease, we compared baseline cavernous sinus gradients to baseline and CRH-stimulated petrosal sinus gradients in 15 patients with surgically proven Cushing disease. Contrary to a recent report, the test sensitivity was only 80% for the baseline cavernous sinus ACTH gradient (3 [20%] patients had a central-to-peripheral gradient < 2 and were thus false negatives) compared to 100% sensitivity for the CRH-stimulated petrosal sinus ACTH gradients (gradients of 26.7, 91.3, and 52.6) in the same patients. We conclude that petrosal sinus sampling with CRH stimulation is a more accurate diagnostic procedure than cavernous sinus sampling without CRH.

4) Cushing syndrome in children and adolescents

Cushing syndrome is rare in children, and thus comprehensive information concerning diagnosis, differential diagnosis, and treatment are difficult to obtain. To address this need, we have analyzed the data from 59 patients with Cushing syndrome between the ages of 4 and 20 who were admitted to NIH during the past decade.

Fifty patients had Cushing disease, 3 had ectopic ACTH production, and 6 had primary adrenal disease (2 with adrenal cancer and 4 with bilateral primary pigmented nodulocortical adrenal disease). Increased body mass index and decreased growth velocity were the most prominent clinical features. Pituitary microadenomas were not seen by MR imaging in 48% of children with Cushing disease. The high-dose dexamethasone suppression test, CRH test, and petrosal sinus sampling had similar diagnostic performance as in adult patients with Cushing syndrome. Transsphenoidal surgery was effective in 48 of 49 patients with Cushing disease.

These data provide a comprehensive, modern approach to the diagnosis and treatment of Cushing syndrome in children and adolescents. They represent the largest, most accurately diagnosed, and most effectively treated pediatric series that has been reported thus far.

c. Complications

We have published two brief reports during the past year to alert clinicians to rare but potentially devastating complications of Cushing syndrome. The first report describes the initial case of spontaneous vulvar necrotizing fasciitis in a middle-aged woman who had a small pimple-like vulvar lesion that evolved rapidly into a near-fatal episode requiring extensive surgical debridement, hyperbaric oxygen, intensive care, and months of recovery. The case illustrates the extraordinary risk posed by the immunosuppressive effect of Cushing syndrome.

The second report describes the initial cases to appear in the literature of spontaneous hemorrhage into ACTH-secreting microadenomas causing Cushing disease. In each case the hemorrhages, which were not recognized clinically at the time of occurrence, caused temporary remission of the Cushing disease with recurrence a year or more afterward owing to renewed growth of the surviving tumor cells. After the hemorrhages, the biochemical profiles of the patients were initially consistent with secondary adrenal insufficiency, which could have led to an incorrect diagnosis of factitious Cushing syndrome. Thus, hemorrhage into ACTH-secreting microadenomas is a potential cause of unexplained remission of Cushing syndrome.

d. Treatment

1) Recovery from osteoporosis after cure of Cushing syndrome

Osteoporosis is a well-recognized complication of Cushing syndrome, although relatively little is known about the natural history of this complication in treated patients. To gain further information about the potential of osteoporotic bone in Cushing syndrome to recover normal bone density after cure of hypercortisolism, patients with Cushing syndrome are undergoing bone density measurements by dual energy x-ray absorptiometry (DEXA) before and after successful treatment. If patients fail to show recovery of bone density within several years after cure, we plan to initiate a therapeutic trial to determine whether bone density can be improved by any of the treatments employed in the treatment of idiopathic or postmenopausal osteoporosis.

2) Mechanism of hypogonadism in Cushing syndrome and effect of successful treatment

Decreased plasma testosterone, impotence, and decreased libido are common in men with Cushing syndrome. Hypogonadism also occurs commonly in women with Cushing syndrome, but is more difficult to study because of the need to control for the stage of the menstrual cycle. To gain further insight into the mechanism of hypogonadism in Cushing syndrome, spontaneous gonadotropin secretion and the gonadotropin response to administration of gonadotropin-releasing hormone are being studied before and after successful treatment.

3) Drug treatment of ACTH-secreting tumors in Cushing disease and in Nelson syndrome

Following bilateral adrenalectomy for Cushing disease, the pituitary tumors sometimes enlarge and secrete markedly increased amounts of ACTH (Nelson syndrome). Although 3 drugs have had limited effectiveness in suppressing the function of these tumors (cyproheptadine, bromocriptine, and valproic acid), none has achieved widespread clinical acceptance because of a low response rate. Since these 3 drugs are believed to act through different mechanisms, we conducted a study to determine whether the simultaneous use of these agents might be additive or synergistic in suppressing ACTH secretion in persistent Cushing disease after unsuccessful transsphenoidal surgery and in Nelson syndrome. With the single-dose study design that was employed, only bromocriptine and the combination of all three agents achieved statistically significant suppression of plasma ACTH. Addition of cyproheptadine and valproic acid did not have any further ACTH-suppressing effect.

Significance:

1) Fetal adrenal zone and adrenarche. Adrenal androgens from fetal adrenal are the major precursors for placental estrogen synthesis during pregnancy, which plays an important role in preparing the breast for lactation and in inducing uterine oxytocin receptors. Postnatally, adrenal androgens play an important pathogenetic role in hormone-dependent cancers such as those of the breast and prostate, and in several endocrine disorders such as congenital adrenal hyperplasia, premature adrenarche, hirsutism, and Cushing syndrome. Increased understanding of the mechanisms regulating adrenal androgens may lead to new methods of controlling adrenal androgen secretion in these disorders.

2) Congenital adrenal hyperplasia (CAH). This is among the most common autosomal recessive disorders in the United States population. The classic form affects approximately 1 in every 13,000 children (about the same as PKU), and has a heterozygote frequency of about 1 in 57. The nonclassical or late-onset form of CAH is even more common. Thus, advances in the treatment of this disorder will benefit the 300 children with classic CAH and the larger number of nonclassical patients born annually in the United States.

3) Hirsutism. Approximately 1 woman per 1000 (a prevalence of nearly 100,000 women in the United States) has hirsutism sufficiently severe to constitute a significant management problem. These women often develop anxieties about their feminine identity that hinder normal social development during the teen-age and adult years. Thus, improved understanding of the pathogenesis of this disorder, and improved treatment methods, could enhance the well-being of a large number of women.

4) Cushing disease. This disorder is estimated to affect 600 to 2000 new cases per year in the United States. When described by Cushing in 1932, it was a devastating, uniformly fatal illness that often struck in the prime of life. Recent advances have greatly improved our ability to diagnose accurately and to treat effectively this potentially lethal disease.

5) Adrenal insufficiency. This potentially fatal disorder continues to pose a therapeutic challenge because of the morbidity associated with undertreatment or overtreatment. Improved methods of treatment are needed to restore these patients to full health.

Proposed Course:

1) The hypothesis that adrenarche results from a proopiomelanocortin (POMC)-derived peptide will be tested by comparing the adrenal androgen response to corticotropin-releasing hormone (CRH), which stimulates POMC, to that of ACTH, which suppresses POMC. This study will be carried out in patients with hypothalamic adrenal insufficiency.

2) Congenital adrenal hyperplasia. Future studies will address the following areas:

a) The effect on growth rate, bone maturation, predicted adult height, and final adult height of a new regimen consisting of antiandrogen (flutamide), aromatase inhibitor (testolactone), reduced hydrocortisone dose, and

fludrocortisone versus a conventional hydrocortisone/fludrocortisone regimen.

b) The effect, in a recently discovered strain of mice with 21-hydroxylase deficiency, of this disorder on the development of the hypothalamic CRH neuron, as determined by immunohistochemistry and in situ hybridization methods.

c) Use of the 21-hydroxylase-deficient mouse to develop approaches to correct the 21-hydroxylase deficiency by gene therapy approaches.

Testing of new therapeutic strategies would benefit from the recruitment of a birth cohort of CAH patients whose potential adult height has not been compromised by inadequate treatment during the first 1-2 years of life. To identify such a cohort, we are attempting to interest the nearby state laboratories responsible for newborn screening in setting up a pilot newborn screening program. Effective, inexpensive methods for newborn screening of CAH have been developed and are in use in Italy, Japan, and the states of Washington, Alaska, Illinois, Texas and other states.

3) Cushing syndrome

To improve the usefulness of inferior petrosal sinus sampling (IPSS) in the differential diagnosis of Cushing disease versus ectopic ACTH secretion, additional patients will be studied with the new DEX/IPSS test. To decrease the time and cost of evaluation of hypercortisolism, a 1-day metyrapone test, dexamethasone test, and oCRH test will be evaluated prospectively. Other initiatives include efforts to define the natural history of the osteoporosis and hypogonadism of Cushing disease before and after successful transsphenoidal microsurgery. If the osteoporosis is not reversible spontaneously with cure of hypercortisolism, new therapeutic strategies will be developed to attempt to prevent future losses of bone density. Additionally, the effectiveness of medical treatment to suppress ACTH-secreting adenomas will be evaluated in Nelson syndrome and in persistent Cushing disease after unsuccessful transsphenoidal surgery.

To gain insight into the molecular basis for the corticotroph neoplasms that cause Cushing disease, RNA from these neoplasms will be amplified by RT-PCR to detect the presence of potential oncogenes. Our ultimate goal is to gain new insights into the molecular basis for this disorder that can be used to design innovative strategies to eradicate these tumors or to inhibit their growth and excessive hormone secretion.

Protocols:

Animal

92-016	Yanovski	Comparison of the density of hypothalamic corticotropin-releasing hormone neurons between normal mice and mice with congenital adrenal hyperplasia
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Human

93-CH-101	Merke	Flutamide and testolactone treatment of children with classic congenital adrenal hyperplasia (21-hydroxylase deficiency)
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This study tests the hypothesis that the regimen of flutamide, testolactone, and reduced hydrocortisone dose will normalize the growth and development of children with congenital adrenal hyperplasia (CAH). The study employs a randomized, open, parallel design comparing the new regimen to conventional therapy for a period of two years.

The preliminary results have shown decreased growth velocity, weight velocity, and bone maturation rate during the new regimen despite the decrease in glucocorticoid dose and increase in the adrenal androgen precursor, 17-hydroxyprogesterone, in plasma. Only one child in 26 has failed to tolerate the new regimen, due to GI

intolerance. No other adverse effects have been observed. We conclude that the new regimen has short-term effectiveness and deserves longer term study.

90-CH-194	Yanovski	Inferior petrosal sinus sampling for the determination of adrenocorticotrophic hormone (ACTH) concentration in normal volunteers and in patients with disorders of the hypothalamic-pituitary-adrenal axis
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This study is currently testing the hypothesis that the 2-day, 8-mg dexamethasone test administered before inferior petrosal sinus sampling (Dex/IPSS test) will suppress normal corticotroph function during the IPSS procedure but will permit continued ACTH secretion from ACTH-secreting pituitary microadenomas causing Cushing disease. If correct, this hypothesis implies that the Dex/IPSS test will extend the diagnostic usefulness of IPSS to patients with intermittent Cushing syndrome, mild Cushing syndrome, or prior adrenal blockade. These are settings in which the conventional IPSS procedure may yield misleading results because central-to-peripheral ACTH gradients arising from normal corticotroph cells may be mistaken for an ACTH-secreting microadenoma. Thus, an erroneous diagnosis of pituitary Cushing disease may be made in the patient with ectopic ACTH syndrome or with an unrecognized pseudo-Cushing state.

The study employs a randomized, open design in which the patient receives 2 IPSS procedures, one with and one without prior 8 mg per day dexamethasone administration for 2 days. The diagnostic performance of the conventional and modified IPSS procedure will then be compared against the "gold standard" diagnosis determined at follow-up (the "gold standard" is surgical cure or pathologic diagnosis for Cushing syndrome and remission or prolonged lack of progression for pseudo-Cushing states).

Preliminary results suggest that the Dex/IPSS procedure has improved diagnostic accuracy compared to IPSS in settings in which normal corticotroph function may not be suppressed. We conclude that further evaluation of this new test is warranted.

94-CH-0144	Asis	Bone mineral density and bone metabolism in patients with untreated and cured endogenous Cushing syndrome
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This study tests the hypothesis that patients with osteoporosis owing to Cushing syndrome will recover normal bone density within 3 years of the cure of Cushing syndrome. We further hypothesize that this recovery of bone density will be attributable to an increased rate of bone formation without a corresponding increase in bone resorption. The study employs a longitudinal, observational design in which each subject's pretreatment bone density, bone formation markers, and bone resorption makers are compared by ANOVA for repeated measures with the corresponding values during the 3 years after the cure of Cushing syndrome.

The available pretreatment results indicate low-turnover osteoporosis before treatment of Cushing syndrome, indicating that both the rate of bone formation and of bone resorption are suppressed. Further observation will be required to determine the changes following the cure of Cushing syndrome.

PUBLICATIONS

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00627-06 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Glycoprotein Hormones: Oligosaccharide Structure and Function

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	D. Blithe	Expert	UG, SME, DEB, NICHD
Others:	M. Nemansky	Visiting Fellow	UG, SME, DEB, NICHD
	E. Moy	Clinical Associate	UG, SME, DEB, NICHD
	C. Lyons	Technician	UG, SME, DEB, NICHD

COOPERATING UNITS (if any)

R. Iles, St. Bartholomew's Hospital Medical College, West Smithfield, London, U.K.; V. Reinhold, Boston University School of Medicine, Boston, MA

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Unit of Glycobiology

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The goal of the project is to increase our understanding of the structure-function relationships and glycobiology of the oligosaccharide moieties on glycoprotein hormones. Currently under investigation are, human chorionic gonadotropin (hCG), gonadotropin β -core fragment, glycoprotein hormone free α subunits associated with pregnancy or malignancy, and pituitary free α subunits secreted throughout the normal menstrual cycle. We have shown that the free α subunit purified from pregnancy urine stimulates secretion of prolactin from primary cultures of human decidual cells in a dose-dependent manner. This finding indicated that free α is a glycoprotein hormone with potential functions that are independent of heterodimeric gonadotropins. We further found that free α can play a role in recruitment of endometrial stromal cells for decidualization. This observation expands the function of the free α subunit into areas of reproductive physiology that occur during the luteal phase of the menstrual cycle and may suggest involvement of free α in implantation.

Carbohydrate modifications, resulting in a variety of branched glycan structures, occur on all glycoproteins prior to secretion. These modifications can affect virtually every aspect of the molecule's behavior, including receptor binding and signal transduction, yet the underlying regulatory mechanisms remain elusive. We have investigated functional aspects of the glycan moieties on free α , demonstrating that specific types of carbohydrate modifications on free α can either prevent or facilitate combination with hCG- β to form intact hormone. We have found that the free α subunits that are located in fetal compartments in high concentrations during early pregnancy exhibit physical properties that are similar to those of the free α subunit isolated from pregnancy urine. We have shown that the oligosaccharide moieties on this early pregnancy free α function to maintain the molecule in an uncombined form in placental compartments that also contain high amounts of combinable free β -subunit.

We have identified differences in the oligosaccharide moieties of free α and hCG as a function of gestational development; in particular, the glycosylation of free α changes dramatically as pregnancy progresses. In five pregnancies examined thus far, the glycosylation changes were found to begin at week 14-15 of gestation and were completed by week 17, suggesting developmental regulation of glycoprocessing enzymes in the cells that secrete α subunit. We plan to examine how the differences observed in glycosylation affect the bioactivity of these molecules.

Project Description

Objectives:

The overall objective of the project is to further our understanding of the structural elements of glycoprotein hormones and how these elements, particularly the carbohydrate moieties, contribute to the function of these molecules. The molecules under study were, human chorionic gonadotropin (hCG), gonadotropin β -core fragment and glycoprotein hormone free α subunit.

Glycoprotein hormone free α subunit is a major placental secretory product during pregnancy. Free α is produced throughout pregnancy by trophoblast cells of the placenta. Although the highest maternal serum levels of free α occur during third trimester, much higher concentrations of free α are observed in the extraembryonic coelomic fluid and the amniotic fluid in early pregnancy. Free α subunits are also secreted by the pituitary throughout the normal menstrual cycle in nonpregnant individuals. Recent structural data indicates that α subunit belongs to a superfamily of growth factors that share structural homology. We have recently described a potential function for free α subunit in pregnancy, demonstrating that it can stimulate secretion of prolactin from primary cultures of human decidual cells. In view of our findings, we propose that free α is a glycoprotein hormone with activity that is independent of the heterodimeric hormones (hCG, LH, FSH and TSH) that contain α subunit in combination with a β subunit. The hypothesis that free α is an independent glycoprotein hormone defines a new area of basic and clinical research on the functions, physiology, and glycobiology of free α molecules.

In view of the dominant role that glycosylation can play in regulating hormonal activity, our goal is to elucidate the structure and function of the carbohydrate moieties on pregnancy-related hormones. Changes in glycosylation of these molecules were examined as a function of gestational development. Knowledge of the oligosaccharide structures on hCG and free α is critical for determining the contribution of specific oligosaccharides to the functions of these molecules. Also, knowledge of the normal structures of these molecules throughout pregnancy is expected to provide information about the regulation of glycosylation during pregnancy and to provide a basis for distinguishing aberrant structures in diseases of pregnancy.

Methods Employed:

Studies on hCG and free α molecules that were secreted at different times during pregnancy were performed on urine samples collected at weekly intervals throughout five individual pregnancies. Extraembryonic coelomic fluid (EECF) was collected via amniocentesis at weeks 7-12 of pregnancy from sixteen volunteers (collection performed at St. Bartholomew's Hospital, London, U.K.). Amounts of hCG, free α , hCG- β subunit, β -core fragment, human prolactin, and IGFBP-1 were assessed by radioimmunoassay using polyclonal or monoclonal antisera. In some cases, purification of hCG from free α was not desirable, therefore, we used monoclonal antibodies in a radioimmunoassay that recognized either intact hCG or free α with minimal cross reactivity. For structural characterization of hCG and free α , the hormones were purified by gel filtration and immunoaffinity chromatography. Carbohydrate structure was determined by lectin affinity chromatography using lectins with a variety of carbohydrate specificities. The lectins used in the current study were Concanavalin A, Lens culinaris, and Datura stramonium. Carbohydrate composition was determined after acid hydrolysis of purified material. Monosaccharides were separated by high pH-anion exchange HPLC and were quantified by pulsed amperometric detection.

For the free α bioassay, primary cell cultures of endometrial stromal cells were established from endometrial biopsies obtained during various times of the menstrual cycle. Cells were incubated in culture media containing a variety of hormonal additions including the presence or absence of progesterone and various doses of α subunit.

A variety of standard biochemical methods were also employed in these studies, including enzymatic digestion, density gradient centrifugation, SDS-polyacrylamide gel electrophoresis, immunoblotting, etc.

Major Findings:

We have recently postulated a novel function for free α in pregnancy. We have shown that the free α molecule purified from pregnancy urine stimulates secretion of prolactin from primary cultures of human decidual cells isolated from term pregnancies. The concentration of free α that stimulated prolactin was well-within the physiologic maternal serum free α levels during pregnancy. Thus, placental free α molecules appear to have a functional role in pregnancy by stimulating decidual prolactin secretion, thereby identifying free α as an independent glycoprotein hormone.

We have now extended our findings to show that free α is likely to play an important role in the normal menstrual cycle and in very early pregnancy. In addition to the fact that free α is a major placental product during pregnancy, free α molecules are also secreted by the pituitary throughout the normal menstrual cycle in a pulsatile fashion that is regulated by GnRH. Using cells obtained by endometrial biopsy, we have found that free α can stimulate endometrial stromal cells to undergo cellular differentiation to become decidualized cells. Decidualized endometrium is characterized by morphologic changes and by secretion of prolactin and IGFBP-I, and the process of decidualization is crucial for development of a uterine lining that is receptive for implantation. Addition of free α in a dose range that is within normal physiologic levels of the menstrual cycle results in morphologic changes associated with decidualization with concomitant stimulation of prolactin and IGFBP-I secretion. The effects of free α are dramatically enhanced by simultaneous addition of progesterone, indicated a synergistic response of the combined hormones compared to the additive effects of either hormone alone. These findings demonstrate that free α is likely to be playing a role in the preparation of the uterus for receiving the developing embryo. This novel concept may have important implications regarding infertility, recurrent spontaneous abortion and assisted reproduction.

We examined whether hCG could produce a stimulatory effect that was similar to the activity observed with free α subunit. At equivalent molar concentrations, intact hCG was unable to stimulate endometrial differentiation either alone or in combination with progesterone. At higher doses of hCG, some stimulation was observed, however, during incubation of hCG with the cells, uncombined α subunit was generated from the hCG preparation. The amount of stimulation that was observed with high concentrations of hCG could be attributed to the uncombined α subunit that was generated during the incubation period. The observation that uncombined α subunit can be generated from hCG preparations and that this α subunit is bioactive may be important in designing optimal hormonal conditions for use in assisted reproduction protocols.

Extraembryonic coelomic fluid (EECF) occupies the space between the amniotic membrane and the chorionic membrane of the developing trophoblast representing a prominent cavity until about 12 weeks of pregnancy. The molar concentration of free α in the EECF is the highest level ever reported for a physiologic compartment, far exceeding the levels in amniotic fluid or maternal serum at the same point of gestational development. In addition to free α , this cavity also contains high concentrations of intact hCG and free β subunit in a molar ratio of 6:3:1, respectively. We have shown previously that modifications to the N-linked glycans of free α occur during glycoprocessing of the molecule resulting in branched glycan structures that can prevent free α from combining with hCG β -subunit. To determine if a similar phenomenon occurred in the placentally-derived subunits of the EECF, we examined the EECF free α and free β populations for their abilities to combine with the complementary dissociated subunit. We showed that the EECF free β was fully capable of combination, whereas nearly all of the EECF free α subunits were unable to combine. However, when we treated the EECF free α with N-glycanase to remove the N-linked oligosaccharides, we could convert the bulk of the free α molecules into combinable subunits. Therefore, we were able to conclude that the N-linked glycans on free α protect it from combining with the available and combinable free β -subunits that coexist in the same placental compartment. Thus, this single modification to the α subunit can protect free populations of both α and β subunits.

We have purified hCG and free α from the EECF and have demonstrated that EECF-free α is larger than the α -subunit found in EECF-hCG. The larger size of the EECF-free α suggests that it is a secreted molecule rather than a product of dissociation or degradation of hCG. We have analyzed the carbohydrate composition of the EECF free α and found that it very closely resembles the overall composition of the free α purified from

pregnancy urine. However, there appear to be substantial proteolytic cleavages in the free α obtained from urine as evidenced by smaller molecular size and a heterogeneity of bands of the reduced material on SDS-PAGE. Furthermore, treatment of the urinary free α with N-glycanase to remove oligosaccharides did not yield an increase in combinable forms of α , suggesting that peptide components necessary for combination may have been damaged or removed in the process of clearance into the urine.

The glycosylation of hCG and free α had been assumed to be unchanged throughout pregnancy, however, we have shown that their glycosylation does change as gestation advances. We have examined the time points between week 12 and week 28, previously designated as early and late pregnancy, respectively, to determine if the changes in glycosylation were gradual throughout gestation, or occurred suddenly at a specific point in gestational development. Particularly on free α , and to a lesser extent on hCG, changes in the branching and fucosylation of the glycans were observed as pregnancy progressed. The glycosylation patterns between individual healthy pregnancies were remarkably similar to one another when the same period of gestation was compared, and all of the individuals displayed similar changes in the glycosylation of free α within a narrow time-frame of early second trimester. It is important to note that similar changes in glycosylation are also observed after malignant transformation, and that alterations in the glycosylation of hCG and α -fetoprotein have been suggested as potential diagnostic markers for malignancy. However, normative patterns for hCG and free α glycosylation throughout uncomplicated pregnancies have not been established. Since there are extensive parallels between malignant transformation and embryonic development, it is crucial to obtain these normative data in order to establish aberrant glycosylation profiles in disease. In each of the five individual pregnancies we have determined that the bulk of the change occurred between weeks 14 and 16. After reaching the glycosylation pattern achieved by week 17, the profile remained constant for the remainder of the pregnancy. It is likely that the changes in the glycosylation patterns reflect alterations in some key glycoprocessing enzymes that are under developmental regulation.

Previously, we had developed a highly specific assay for measuring the β -core fragment of hCG in the presence of structurally similar molecules such as β -subunit and intact hCG. Elevated levels of β -core had been observed in a number of malignancies and we had postulated that our assay might have clinical utility in identification of abnormal conditions. Recently, Iles and colleagues had observed that β -core levels were elevated in pregnancies affected by Down's Syndrome, however, their initial observations were limited to a small number of samples. We are currently participating in a multi-center study to evaluate the potential utility of gonadotropin β -core fragment as a marker of pregnancy affected by Down's Syndrome. In a blinded study of 373 samples, using a cutoff of 95% we were able identify 13 of 22 (64%) pregnancies affected with Down's Syndrome using our β -core assay. If this detection rate can be sustained in a larger prospective study, β -core would be the best single biochemical indicator currently available. Measurement of β -core is performed as a non-invasive urine test and the results could potentially be combined with other indicators to yield a very high rate of detection that would identify a population that could undergo amniocentesis to confirm the diagnosis. The ability to detect the elevated β -core levels associated with Down's Syndrome did not appear to be affected by maternal age. Thus, this non-invasive test may be useful as a screening device for pregnancies that would be considered at low risk for the chromosomal abnormality.

Significance to Biomedical Research and the Program of the Institute:

In spite of the copious production of gonadotropin free α subunit in pregnancy, no biological role had been established for it. We have now identified two important activities of free α . Our earlier studies demonstrated that free α could stimulate prolactin secretion from terminally differentiated decidual cells suggesting that placental free α functions in the maintenance of an established pregnancy. Our most recent experiments indicate that free α can work synergistically with progesterone to induce decidualization of uterine endometrial stromal cells in culture. These results imply that free α secreted by the pituitary plays a role in preparation of the uterus for implantation. Thus, free α is a glycoprotein hormone with bioactivity that occurs during the normal menstrual cycle and it is likely that free α also has endocrine and paracrine functions during very early pregnancy when it is being secreted by trophoblast cells of the developing placenta. This novel concept will require expansion of the scope of endocrinological investigations to include potential contributions from free α , in addition to those

of the heterodimeric members of the gonadotropin family. Elucidation of the functions of free α may have important implications for certain cases of infertility, luteal phase defects, or recurrent miscarriage. Furthermore, closer monitoring of the free α subunit levels in procedures used for assisted reproduction may be necessary. It is conceivable that free α may have a role in implantation such that too much or too little free α could be undesirable. Additionally, our discovery that free α acts synergistically with progesterone has important implications for timing events that might control the implantation window.

We previously had compared the carbohydrate structures on the free α molecule in pregnancy with those on the hCG- α subunit isolated after dissociation of intact hCG. Although the two α molecules contain the same polypeptide structure, we showed that they have distinctly different carbohydrate moieties and that these carbohydrate structures could either prohibit or facilitate combination with hCG- β subunit to form intact hCG. In normal pregnancy, the vast majority of α polypeptides that are synthesized during the second and third trimesters of pregnancy become free α molecules. The recent finding that in the first trimester, the EECF compartment contains amounts of free α that are higher than those of hCG on a molar basis suggests that there may be a role for free α in early pregnancy. Our finding that this EECF-free α is larger than the α subunit contained within the intact EECF-hCG molecule implies that, like the free α that we have isolated from pregnancy urine, EECF-free α is synthesized with carbohydrate modifications that prevent combination with β -subunit. Since β -subunit is also present in substantial amounts in the EECF, it may be more crucial to prevent post-secretory combination of α and β in the EECF or amniotic fluid than in maternal serum where β -subunit represents a very minor component. The larger size of the EECF-free α also strongly argues against the hypothesis that the free subunits could originate from dissociation of nicked forms of hCG.

The glycosylation of these molecules had been assumed to be unchanged throughout pregnancy, however, we have now shown that glycosylation changes dramatically as gestation advances. We have established that this change in the glycosylation profile of free α occurs during a relatively narrow window of gestation, between weeks 14 and 17 of pregnancy. Having demonstrated that there are developmentally associated changes in the glycosylation of free α , it may be possible to determine the specific glycoprocessing enzymes involved and to identify the regulatory elements for those enzymes.

It is important to note that similar changes in glycosylation branching and fucosylation are also observed after malignant transformation, and alterations in glycosylation of hCG and α -fetoprotein have been suggested as potential diagnostic markers for malignancy. However, normative patterns for glycosylation of these molecules throughout uncomplicated pregnancies have not been established. Since there are extensive parallels between malignant transformation and embryonic development, it is crucial to obtain these normative data in order to establish aberrant glycosylation profiles in disease.

We have previously developed a highly specific assay for measuring the β -core fragment of hCG in the presence of structurally similar molecules such as β -subunit and intact hCG. Recently, Iles and colleagues had suggested that β -core levels might be elevated in pregnancies affected by Down's Syndrome, however, their initial observations were limited to a small number of samples. We are currently participating in a multi-center study to evaluate the potential utility of gonadotropin β -core fragment as a marker of pregnancy affected by Down's Syndrome. Using our β -core assay, we were able to detect 64% of Down's-affected pregnancies with a false positive rate of 5%. If this detection rate can be sustained in a larger prospective study, β -core would be the best single predictor currently available. Measurement of β -core is performed as a non-invasive urine test and the results could potentially be combined with other indicators to yield a very high rate of detection that would identify a population that could undergo the more invasive techniques to confirm the diagnosis. This screening test has the potential to diagnose Down's-affected pregnancies regardless of maternal age.

Proposed Research

We have described preliminary findings indicating that free α is likely to be a glycoprotein hormone with activity that is independent of hCG. This novel concept will require expansion of the scope of endocrinological investigations to include potential contributions from free α , in addition to those of the other members of the

gonadotropin family. The goal of the current research is to elucidate the function and glycobiology of free α in reproduction and development. The specific aims are divided into three main areas: A) To investigate the hormonal functions of free α , in particular, to define the role of free α in the regulation of human decidual function in pregnancy, in cellular differentiation in the normal menstrual cycle and in cellular differentiation during fetal pituitary development; B) To elucidate the structure and function of carbohydrate moieties on free α and hCG, and to ascertain the nature and the role of changes in the glycosylation of these molecules that occur as a function of gestational age; C) to evaluate the clinical utility of gonadotropin β -core fragment as a marker for clinical problems, in particular, as a screen for Down's syndrome or for early detection of recurrence of malignancy.

We will pursue studies to elucidate the function of free α as an independent hormone. Having demonstrated that free α evokes a response from decidual cells, we plan to show that the response is a receptor-mediated phenomenon. We will perform binding studies on cells obtained from endometrial biopsies to characterize the putative receptor. We will investigate the potential functions of free α in fetal pituitary lactotrope differentiation using a recently developed transgenic mouse lacking α subunit.

We are continuing our structural characterization of the changes that occur on hCG and free α obtained from early and late pregnancy. We have identified a narrow gestational window in which the glycosylation changes occur. We will use sensitive mass spectrometry techniques to determine the explicit structural changes that are occurring during this time period. These structures will pinpoint specific enzymes which may be targets of developmental regulation. We will determine if the developmental changes in the glycosylation of free α are found at both glycosylation sites or predominate at a single site. Also, having demonstrated that there are dramatic structural differences on free α between early and late pregnancy, it is important to examine how these structures contribute to the various bioactivities of free α .

We will continue to participate in a multicenter study to evaluate the potential clinical utility of β -core fragment levels as a marker for Down's syndrome in pregnancy. We will also screen patients who have had surgery to remove hCG-producing tumors, to determine if urinary β -core levels might detect recurrence of a malignancy prior to observations of hCG in serum.

Protocols:

93-CH-0075	Blithe	The role of gonadotropin free α subunit in the regulation of decidual cell function.
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The goal of this protocol is to investigate the hormonal function of gonadotropin free α subunit, in particular, to define the role of free α in the regulation of human decidual function. We are exploring a possible role for gonadotropin free α subunit in the differentiation of endometrial stromal cells obtained from endometrial biopsies performed at mid-cycle when decidualization may begin to occur.

B-93-009(NNMC)	Nash	The role of glycoprotein hormone alpha subunit in the regulation of human decidual function in pregnancy.
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The goal of this protocol is to investigate the hormonal function of gonadotropin free α subunit in pregnancy, in particular, to define the role of free α in the regulation of human decidual function. We are exploring a possible role for gonadotropin free α subunit in stimulation of prolactin secretion from decidual cells obtained from term pregnancy placental membranes.

Publications:

Blithe DL, Iles RK. The role of glycosylation in regulating glycoprotein hormone free alpha and free beta subunit combination in the extra-embryonic coelomic fluid of early pregnancy, *Endocrinology*, 1995;136:903-10.

Kraiem Z, Sadeh O, Blithe DL, Nisula BC. Human chorionic gonadotropin stimulates hormone secretion, iodide uptake, organification and cAMP formation in cultured human thyrocytes, J Clin Endocrinol Metab, 1994;79:595-9.

Thotokura NR, Blithe DL Glycoprotein hormones: glycobiology of gonadotropins, thyrotropin and free alpha subunit, Glycobiology 1995;5:3-10.

Patents:

Blithe DL, Wehmann RE, Nisula BC. U.S. Patent 5, 445, 968: Purification of human chorionic gonadotropin β -core molecule and preparation of antibodies with specificity for same, August 29, 1995. A product from this application, a β -core detection kit, has been licensed and marketed by Triton Laboratories, a division of Ciba Corning Diagnostics Corporation.

U.S. Serial No. 08-448,079; Blithe, D.L., Wehmann, R.E. and Nisula, B.C. Purification of human chorionic gonadotropin β -core molecule and preparation of antibodies with specificity for same. Continuation-in-part of U.S. Serial No. 07-292,985. Foreign filing license granted. Patent pending.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00628-06 DEB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Biological roles and mechanisms of action of insulin-like growth factors (IGFs)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	C.A. Bondy	Senior Clinical Investigator	SGM, DEB, NICHD
Others:	O. Adesanya	Clinical Associate	SGM, DEB, NICHD
	E. Chin	Senior Staff Fellow	SGM, DEB, NICHD
	C. Mitchell	Pre-IRTA Fellow	SGM, DEB, NICHD
	R. Reinhardt	Clinical Associate	SGM, DEB, NICHD
	E. Wang	Senior Staff Fellow	SGM, DEB, NICHD
	J. Wang	Special Volunteer	SGM, DEB, NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Growth and Metabolism

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

6.0

PROFESSIONAL:

5.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This Section carries out research on the biological role and mechanisms of action of insulin-like growth factors -I and -II (IGFs-I and -II). These particular growth factors have major roles in development as proven by the fact that targeted deletion of IGF-I and IGF-II gene expression results in severe dwarfism and, in the case of IGF-I, 90% mortality— although how IGF deletion causes these defects is not yet understood. Molecular, immunological and radioligand probes are used to analyze the cellular sites of synthesis and action of these growth factors, their putative regulators and potential targets during embryonic development as well as in different physiological and pathophysiological situations in which IGFs have key roles.

This Section has made progress over the past year in dissecting the signal transduction pathways and biological functions of IGFs in the brain. A specific protease termed IDE has been implicated in selective termination of IGF induced signalling and two distinct cGMP-inhibited phosphodiesterase isoforms (PDE3A and PDE3B) have been implicated in IGF signal transmission in the developing and mature brain, respectively. Spatiotemporal patterns of IGF system expression have been documented in the developing chick brain, establishing the fundamental conservation of expression patterns and probable roles in avian and mammalian species. Additional studies have implicated specific IGF system responses in recovery from acute and chronic ischemia and a variety of demyelinating diatheses.

The group has defined the mechanism by which IGF-I promotes ovarian follicular selection, showing that this intrinsically produced peptide stimulates granulosa cell division and FSH receptor expression, thus regulating gonadotropin responsiveness and ultimately estrogen production and ovulation. A non-human primate model is being developed to study the effects of different menopausal hormone replacement therapy regimens on local growth factor production and tissue hyperplasia in female reproductive organs. A major new finding is that the addition of progesterone to estrogen treatment produces a drastic upregulation of IGF-I in the primate myometrium which is strongly correlated with myometrial proliferation. These data are predicted to have major impact with regard to the design of hormone replacement therapy regimen for women at risk for myometrial tumors and for treatment of leiomyomas.

PROJECT DESCRIPTION:Objectives/Methods:

The goal of this project is to elucidate the biological roles and mechanisms of action of insulin-like growth factors-I and -II (IGF-I and -II). A very fruitful approach to unfolding the IGF story has been investigation of the microenvironmental context in which the peptides, the different receptors and binding proteins are expressed in vivo, both during the course of normal development and in response to experimentally induced and naturally occurring perturbations. Information about the cell types expressing the different components of the IGF system, physiological changes in levels of gene expression in these different cell types and the nature of cellular functions associated with IGF expression provides insight into IGF's physiological roles in a number of different settings. In vitro studies have identified putative regulators of IGF expression and molecular components implicated in IGF-induced signal transduction and these entities are investigated with respect to their in vivo involvement in IGF system expression and function. The ultimate goal is to apply knowledge of IGF's biological roles in the diagnosis and treatment of human developmental and growth disorders and disease.

Major findings:1) Molecular mechanisms of IGF action in the nervous system

a) Relationship to insulin degrading enzyme and cGMP-inhibited phosphodiesterases.

Insulin-degrading enzyme (IDE) is a serine protease linked specifically to the degradation and hence signal termination for insulin. To determine if IDE could also be instrumental in proteolysis and termination of IGF signaling in the CNS, we compared the ontogeny and neuroanatomical expression at the cellular level of IDE and the IGF-I/II receptor and cognate ligands. There is in fact a striking correlation between IDE and IGF-I receptor expression at all stages of development, suggesting that following internalization of the peptide-receptor complex, IDE-catalyzed degradation of the peptide is a mechanism to regulate the duration of IGF action.

Further studies were aimed at the elucidation of signal transduction pathways of IGFs in the CNS. Cyclic GMP-inhibited phosphodiesterases are important regulators of intracellular cAMP levels and are uniquely sensitive to inhibition by cGMP. They are also distinct from other phosphodiesterase species in that they are SELECTIVELY REGULATED BY INSULIN. This phosphodiesterase family contains two different isoforms (PDE3A & 3B) encoded by distinct genes and serving tissue-specific roles in regulation of lipolysis, glycogenolysis, myocardial contractility, and smooth muscle relaxation. IGF-I has been shown to stimulate PDE3 activity leading to a significant reduction in cAMP levels in various cell types, suggesting that this class of enzyme is important in IGF-mediated signal transduction. To determine if one or both of these isoforms could be involved in IGF signal transmission in brain, we compared cellular patterns of PDE3 A&B with IGF system expression in the developing and mature rat brain. The two cGMP-inhibited phosphodiesterase isoforms show distinctive patterns of gene expression; PDE3B expression is similar to the IGF-I receptor while PDE3A expression demonstrates a high level of spatiotemporal heterogeneity and parallelism with local IGF-I expression, suggesting that this isoform is more highly regulated and subserves a variety of developmental stage- and system-specific functions.

The striking parallelism between PDE3A and IGF-I expression and between PDE3B and IGF-I receptor expression supports the view that these enzymes are involved in IGF signal transduction in the developing and mature brain. In general, the high degree of relevance to IGFs noted for molecules originally identified as mediators of insulin action support the view that IGFs and insulin act in fundamentally similar ways.

b) IGFs - comparative neuroanatomy

To determine if our hypotheses derived primarily from the study of murine species concerning the fundamental role of the IGF system in brain development were more broadly relevant across very different species, we investigated cellular patterns of IGF system gene expression in the developing chick CNS. Specific cDNAs encoding chicken IGF-I and insulin and cognate receptors were obtained and used to generate probes for in situ and solution hybridization analysis of brain and retinal development from E2 to E20. Patterns of expression for both receptors were found to be identical to those we previously documented in the rat. IGF-II is concentrated in brain vasculature, meninges and choroid plexus, as in all other species and is also concentrated in projection neurons during a late stage of neuronal development as I have previously described for IGF-I in the rat. Heterologous probes for IGFBP2 and 5 were also used revealing patterns of neuroepithelial expression for these two modulators of IGF action similar or identical to those seen in the rodent. Thus, it appears that IGF system may play a similar role in CNS development in diverse murine and avian species, supporting the view of a highly conserved, fundamental role for IGFs in brain development.

c) IGFs in CNS injury responses

We first demonstrated that there is a strong induction of IGF system expression in response to acute ischemia and also in the local responses to demyelinating insults several years ago. Subsequently, in collaboration with a number of other groups, we have further delineated the time course and cell-type specificity of these IGF system responses to neural injury. With regard to IGF system involvement in recovery from demyelinating insults, important new findings indicate that astrocytic production of IGFBP2 together with IGF-I is necessary for myelin regeneration.

d) IGFs cross the blood brain barrier

In vivo intra-carotid artery infusions were employed to compare amounts and targets of radiolabelled IGF-I, -II and insulin crossing the blood brain barrier (BBB). Results suggest that IGF-I and -II cross the BBB significantly more readily than insulin, and that they are selectively targeted to certain brain sites, most notably the PVN and mediodorsal nucleus of the thalamus, while insulin is largely trapped in the cerebral vasculature. The mechanism by which IGF's are selectively transported into the brain parenchyma appears to involve one of the high-affinity IGF binding proteins, IGFBP2.

2. IGFs in skeletogenesis

The cellular pattern of IGF system in developing cartilage and bone was documented in the rat and mouse. Developing early chondroblasts identified by collagen II gene expression have high level IGF-I receptor, IGF-II and IGFBP5 gene expression; IGF receptor and IGF-II remain high during chondrocyte development and differentiation well into the postnatal period, but IGFBP5 expression is lost as chondrocytes mature. Surprisingly in view of previous reports, IGF-I mRNA is not detected in developing cartilage. IGF-I and other system components are not detected in developing skeleton until the onset of ossification. IGFBP3 mRNA is highly abundant in sprouting capillaries invading the periosteum and eventually forming the marrow cavity and IGF-I, IGFBP4 and 5 mRNAs are all present in osteoblasts. In postnatal day 25 long bones, gene expression for the IGF system is disposed as follows: IGF-II in articular and growth plate chondrocytes and in the periosteum; IGF-I, IGFBP4 and 5 in osteoblasts; IGFBP3 in periosteal and marrow cavity capillary endothelium and IGFBP2 in scattered marrow cells, but never in cartilage or bone cells.

These results challenge the established view that endogenous IGF-I production is critical for chondrogenesis and suggest, instead, that IGF-II is predominant in chondrogenesis and IGF-I in osteogenesis. In addition, our data indicate a transient relationship between IGFBP5 and IGF-II in an early stage of chondroblast development.

Finally, we provide evidence for a functional interaction between IGF-I and IGFBP 4 & 5 expression in osteoblasts involved in both endochondral and membranous ossification.

3) IGFs in diabetic nephropathy

A streptozotocin-induced model of diabetes mellitus in rats was used to investigate the potential role of the IGF system in generating or perpetuating diabetic nephropathy. Profound changes were seen in both gene and protein expression of IGF-I, IGF-I receptor and 3 of the 6 IGF binding proteins. The spatiotemporal patterns of these changes correlate with morphological changes in renal cortex (i.e., thickening of glomerula basement membrane, hypertrophy of proximal tubule segments) and changes in renal function (i.e., increased proteinuria), strongly implicating intrinsic renal IGF system dysregulation in the pathogenesis of diabetic nephropathy. Clinical correlation with human materials is now required to extend these exciting findings.

4. IGFs and the reproductive system

a) Ovary

We have previously reported that IGF-I mRNA is restricted to granulosa cells in a subset of preovulatory follicles in the ovary. To elucidate the follicular correlates of IGF-I expression, we analyzed IGF-I positive follicles with respect to bromodeoxyuridine (BRDU) incorporation, FSH receptor (FSHR) mRNA, c-fos and c-jun immunoreactivity in serial sections from prepubertal and random-cycling mature rat and mouse ovaries. BRDU incorporation and FSHR mRNA are selectively and exclusively detected in IGF-I-positive follicles, while neither c-fos nor c-jun are detected in these same follicles. Spearman correlation analysis of more than 70 individual follicles yielded highly significant positive correlations between IGF-I and BRDU and negative correlations for IGF-I and c-fos/jun with $p < 0.001$ for both analyses; these results showed independence of pubertal or estrus cycle status. Both c-fos and c-jun immunoreactivities are concentrated in corpora luteal granulosa cell nuclei and c-jun alone is concentrated in apoptotic granulosa cell and theca-interstitial cell nuclei. We also investigated IGF-I and FSHR expression in ovarian follicles from hypophysectomized (Hx) and hormone-replaced rats, as well as normal and IGF-I null mice. Follicular IGF-I and FSHR mRNA levels were unaltered in the Hx model but FSHR mRNA levels were below the limits of detection in the IGF-I null mouse ovary, suggesting that pituitary-independent follicular IGF-I production regulates granulosa cell FSHR gene expression.

In summary: 1) There is a highly significant positive correlation between granulosa cell IGF-I and FSHR gene expression and follicular growth, as measured by BRDU incorporation, in the murine ovary. 2) Studies in prepubertal and Hx animals have excluded the possibility that FSH regulates follicular IGF-I production whereas data from IGF-I null mice strongly suggests that IGF-I, directly or indirectly, stimulates granulosa cell FSH receptor expression and thus possibly follicular growth in response to FSH. 3) Cellular patterns of fos and jun expression in the ovary implicate the fos/jun heterodimer in granulosa cell luteinization, while expression of c-jun in the absence of c-fos is implicated in programmed granulosa cell death.

b) Uterus

We have previously documented cellular patterns of IGF system expression in the human ovary, testis and endometrium and established that the rodent is not a good model for local IGF system cellular patterns of expression or regulation as it exists in the human. Because of the difficulty working with human tissues, we are developing a non-human primate model to have better experimental control and more readily available sample materials.

To investigate the role of locally produced insulin-like growth factors (IGFs) in sex steroid induced growth in the primate uterus, ovariectomized rhesus monkeys were treated with placebo (control), estradiol (E2)

alone or estradiol plus progesterone (E&P). After two weeks uteri were removed and serial uterine sections were analyzed by in situ hybridization for IGF-I, IGF-II and IGF-I and -II receptor mRNAs and by immunocytochemistry for detection of the cell proliferation antigen Ki-67. IGF-I and IGF-II and both IGF receptor mRNAs are co-expressed by smooth muscle cells, supporting the possibility for autocrine/paracrine IGF action in stimulating myometrial growth. IGF-I mRNA is barely detected in control myometrium, is significantly increased by E2 treatment and is augmented even more by combination E&P treatment, while little change is noted in IGF-II or IGF receptor mRNA levels. Ki-67 positive myometrial nuclei are also significantly increased by E2 and are augmented more by E&P treatment, with a correlation between local IGF-I mRNA concentration and local Ki-67 positive cell count of $r = 0.89$ ($p < 0.001$). These data provide the first experimental evidence for regulation of IGF-I gene expression by sex steroids in the primate uterus and implicate local IGF-I production in both estrogen- and progesterone-induced myometrial growth.

Significance to Biomedical Research and the Program of the ICD:

Our analysis of cellular and metabolic patterns of IGF system expression in diverse systems during normal development and in response to a variety of physiological stimuli suggests that locally synthesized IGFs function as autocrine or paracrine anabolic boosters, providing— in some as yet poorly understood fashion— for more efficient extraction and/or utilization of energy substrates and building blocks by IGF-expressing cells. These cells are thus able to divide more rapidly, grow bigger or work harder than surrounding cells, with the specific direction of the anabolic response in each case being determined by the baseline state of the cell and local contextual factors.

Proposed Course:

The major thrust of our work over the next few years is directed at testing the validity of this view of IGFs' biological role and elucidating the factors which regulate IGF expression and the mechanisms by which IGFs act to produce their anabolic effects in vivo. Specifically:

Tissue- and developmental stage-specific hypotheses as to IGF anabolic functions will be evaluated by analyzing the morphological and functional consequences of IGF-I and -II deletions in the nervous, reproductive and renal systems of targeted deletant mice (developed by Dr. A. Efstradiatis and now being bred in our lab).

Histological mapping studies will be expanded to investigate what specific metabolic signals might be implicated in inducing IGF expression and what energy-aggrandizing factors may, in turn, be induced by IGFs, and by which signal transduction pathways.

A major initiative on this project over the next few years will establish a novel model system in the Rhesus monkey for analysis of the effects of clinically important hormonal treatments on growth characteristics and hence potential cancer risk of mammary and uterine tissues. The autocrine/paracrine actions of insulin-like growth factors (IGF-I and -II) are implicated in both normal and neoplastic growth of breast and uterus and these factors, at least in human cell lines, are potently regulated by sex steroids. Preliminary data show that cell-specific regulation of IGF expression by estrogen and progesterone contributes to the differential growth effects demonstrated by these sex steroids in reproductive tissues. Thus, an expanded study is proposed to fully evaluate the effects of estrogen, progesterone and tamoxifen on IGF system expression in mammary and uterine tissues. Hormonally-induced changes in the level of IGF, IGF receptor and IGF binding protein expression in specific cell types will be quantitated and statistically compared to the local mitotic index to assess the impact of IGF changes on local growth. Hormone treatments which increase IGF and IGF receptor or decrease inhibitory IGF binding protein expression are predicted to stimulate local hyperplasia and thus provide fertile ground for the emergence and support of neoplastic growth. These studies will elucidate the basic biology of growth regulation in primate mammary and uterine tissues and identify those hormonal treatment regimens which may predispose to cancer

risk thus allowing the rational development of hormone regimens designed to avoid these risks.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00631-06 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Neuroendocrine Control of the Stress Response

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Section on Endocrine Physiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

3.8

PROFESSIONAL:

3.8

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has focused on the mechanism of regulation of hypothalamic and pituitary function during stress. Increases in vasopressin (VP) expression in parvocellular neurons of the PVN, as well as pituitary VP (V1b) receptor upregulation are important determinants of the increased ACTH responsiveness observed in several chronic stress paradigms. Further evidence for the importance of VP was provided by studies during adjuvant induced arthritis in rats, showing elevation in VP mRNA in the parvocellular PVN and increases in pituitary VP receptors accompanying marked activation of the hypothalamic-pituitary-adrenal (HPA) axis.

Studies on the regulation of pituitary VP receptors were extended to investigate the mechanism of regulation of V1b receptor expression. Adrenalectomy causes parallel decreases in VP binding and V1b receptor mRNA, an effect which was prevented by glucocorticoid replacement. Similar changes following adrenalectomy were found in Brattleboro VP deficient rats indicating that the decreased V1b receptor expression was not due to adrenalectomy-induced increases in parvocellular VP. Increasing pituitary exposure VP by surgical shunting of magnocellular VP to the hypophyseal portal circulation caused pituitary VP receptor downregulation without changes in V1b receptor mRNA levels. Dexamethasone administration in intact rats was without effect on V1b receptor mRNA levels. These data suggest that V1b receptor mRNA levels are directly regulated by glucocorticoids or by hypothalamic factors other than VP.

Studies to elucidate the mechanism by which stress induces CRH receptors in the hypothalamic paraventricular nucleus (PVN) focused on the role of glucocorticoids and neural pathways. While adrenalectomy transiently induced CRH receptors in the parvocellular PVN, glucocorticoid administration or withdrawal had no effect on stress induced receptor expression. Surgical hemisections of ascending neural pathways induced CRH receptor mRNA in lower brainstem/spinal cord projecting neurons of the PVN, but had no effect on basal or stress-induced CRH receptor mRNA in hypophyseotrophic parvocellular subdivision. These data show that while glucocorticoids and neural inputs from the brain stem and spinal cord influence CRH receptor expression in the PVN, other factors and neural pathways are responsible for stress-induced CRH receptor expression.

Project Description:Objectives:

The aim of this project is to characterize the neuroendocrine components of the adaptation to stress, with special emphasis in the mechanism of regulation of the hypothalamic-pituitary-adrenal axis. This project involves characterization of receptors for the regulatory hormones, elucidation of the mechanism that regulate expression, synthesis and secretion of hypothalamic regulatory factors, elucidation of the molecular mechanisms that follow hormone receptor interaction, analysis of hormonal secretory patterns and CRF receptor regulation during development and manipulations of the hypothalamic-pituitary-adrenal axis. It is expected that this work will provide information on the physiological regulation of ACTH secretion and provide a basis for the understanding of pathological alterations of the hypothalamic-pituitary-adrenal axis.

Methods employed:

The experiments are performed mainly in rats, and include both in vivo and in vitro procedures. In vivo experiments include application of various stress paradigms, prolonged infusions of CRH, VP and other regulators with osmotic minipumps, blood collection in conscious rats implanted with chronic intravascular catheters, implantation of icv cannulae and lesions of specific brain areas using stereotaxic procedures. In vitro procedures include measurement of CRH, VP and glucocorticoid receptors by conventional receptor assays and autoradiography, detection of neuropeptides by RIA and immunohistochemical techniques, preparation of enzyme-dispersed pituitary and brain cells, measurement of hormone release and intracellular messengers, measurement of adenylate cyclase and protein kinases, measurement of mRNA for several peptides, receptors and enzymes by northern blot, hybridization in solution and in situ, and molecular cloning techniques.

Progress Report:

Previous studies of this laboratory demonstrated three types of responses of the hypothalamic-pituitary-adrenal axis during adaptation to chronic stress: a) desensitization of the ACTH response to the primary stress, but hyperresponsiveness to a novel stimulus, b) sustained ACTH response to the primary stimulus and hyperresponsiveness to a novel stimulus, and c) minor ACTH responses to the primary stimulus and reduced sensitivity to a novel stress. Chronic stress paradigms associated with pituitary hyperresponsiveness showed activation of parvocellular CRH and CRH/VP neurons of the PVN, and increased VP/CRH secretion ratios. The activity of magnocellular neurons which provide VP to the neurohypophysis and peripheral circulation is unaffected by these stress paradigms.

The role of hypothalamic CRH and VP in the regulation of the HPA axis was further studied during development of adjuvant induced arthritis in the rat, an experimental model associated with activation of the HPA axis. irVP in pituitary portal blood increased immediately preceding the onset of arthritis and increases progressively peaking at day 16. The increase in VP is associated with a significant increase in the expression of VP but not CRH mRNA in the medial parvocellular division of the PVN of arthritic rats. In the presence of maximal inflammation there was a decrease in CRH receptor content in the pituitary, whereas VP receptor concentration was significantly increased. Basal and CRH plus VP-stimulated ACTH secretion in vitro was higher in arthritic rats. The results are consistent with the view that activation of the parvocellular vasopressinergic system has an important role in the adaptation of the HPA axis to experimentally-induced chronic stress or arthritis.

Shunting of magnocellular VP and oxytocin (OT) to the pituitary portal circulation by surgical pituitary stalk compression (PSC) was used as an experimental model to study the effects of increasing corticotroph exposure to VP. The consequences of PSC on hypothalamic and pituitary function were studied by in situ hybridization analysis of hypothalamic VP, OT and CRH mRNA levels and pituitary POMC, CRH receptor and VP V1b receptor mRNA levels in rats, with or without ddAVP infusion. 7 days after PSC, there was a marked

decrease in VP mRNA (83%) and a moderate decrease in OT mRNA (38%) in the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei, probably due to retrograde degeneration of magnocellular neurons. In addition, CRH mRNA levels in the parvicellular PVN were decreased by 55% in PSC rats. Correction of diabetes insipidus and osmotic alterations by minipump infusion of ddAVP, 5 ng/hr, s.c., caused a further decrease in hypothalamic levels of VP and OT mRNA (100% and 60%, respectively), but prevented the decrease in CRH mRNA in the PVN. On the other hand, central minipump infusion of VP, up to 3 ng/hr, i.c.v., for 7 days, failed to decrease CRH mRNA levels in the PVN. In the pituitary, POMC mRNA levels were unchanged in the anterior lobe, but were markedly increased in the intermediate lobe, consistent with dopaminergic denervation of the posterior pituitary. In contrast to the decrease in receptor binding observed in PSC rats, mRNA levels for CRH and V1b receptors were similar to control rats, suggesting that receptor downregulation is due to post-translational events. The data show that reduced hypothalamic CRH expression contributes to inhibition of the HPA axis associated with PSC. The decreases in CRH mRNA levels are not due to increased magnocellular activity or elevations in central VP levels, but may involve afferent inputs to the PVN in response to osmotic stimulation, or feedback mechanisms secondary to pituitary overstimulation by VP.

Previous studies of this laboratory have shown that during chronic stress, pituitary vasopressin receptor (V1b) binding and V1b receptor mRNA undergo changes parallel to the changes in responsiveness of the hypothalamic-pituitary-adrenal axis. Further studies were conducted to investigate the mechanisms of regulation of pituitary V1b receptors, the changes in V1b receptor mRNA levels following adrenalectomy and glucocorticoid administration in the rat using Northern blots. Hybridization of pituitary poly[A]RNA was performed using a 363 random primed ³²P-labeled probe corresponding to the coding region of the rat V1b receptor cDNA, known to reveal two mRNA populations with molecular sizes of 3.7 and 3.2 kb. Consistent with the decreases in pituitary VP binding, adrenalectomy caused sustained decreases in both V1b receptor mRNA populations. After 18 hr adrenalectomy, pituitary V1b receptor mRNA levels were reduced by 77% (3.7 kb band) and 62% (3.2 kb band), and after 7 days remained decreased by 65% (3.7 kb band) and 46% (3.2 kb band). Glucocorticoid replacement by injection of dexamethasone, 100 µg, s.c. daily, prevented the inhibitory effect of adrenalectomy. Dexamethasone administration in intact rats resulted in a significant 37% increase in the 3.7 kb V1b receptor mRNA only after 7 day injections. In Brattleboro rats (di/di), which lack hypothalamic VP, pituitary V1b receptor mRNA levels were also decreased after 7 days adrenalectomy, with reductions of 75% and 62% for the 3.7 kb and 3.2 kb bands, respectively, and this effect was prevented by dexamethasone injection. The parallel changes in VP binding and V1b receptor mRNA following adrenalectomy suggesting that regulation of mRNA levels is an important site of control of pituitary VP receptor levels. Although increased VP following adrenalectomy may contribute to the reduced V1b mRNA levels, the results in the Brattleboro rat indicate that V1b mRNA levels are directly regulated by glucocorticoids or by hypothalamic factors other than VP.

Work of this laboratory has shown a stress-specific induction of hypothalamic CRH receptors, with increases in the parvicellular PVN during physical-psychological stress, and in the magnocellular PVN and SON after osmotic stimulation. These studies were extended to study the effect of glucocorticoids and afferent neural pathways on the regulation of CRH receptor expression in the PVN.

In situ hybridization studies showed that adrenalectomy transiently increased CRH receptor mRNA expression in the PVN, from near undetectable levels in controls to 56.4 ± 4.5 (transmittance values) after 18 hr adrenalectomy, an effect which was prevented by glucocorticoid replacement. Longer term adrenalectomy had no effect on CRH receptor mRNA levels in the PVN (2.1 ± 1.5 and 2.0 ± 0.7 , after 4 and 6 days, respectively). In intact rats, 4 hr after immobilization for one hr or a single i.p. hypertonic saline injection, CRH receptor mRNA in the PVN markedly increased ($p < 0.01$), an effect which was unchanged by adrenalectomy (4 or 6 days) or by dexamethasone injection, 100 µg at -14 and 50 µg at -1 hr, prior to stress. The data show that while stress-stimulation of CRH mRNA in the PVN is glucocorticoid-independent, basal levels are likely to be under dual, transcriptional and post-transcriptional control by glucocorticoids.

The role of afferent innervation on CRH mRNA and CRH receptor mRNA levels in the PVN was studied in rats under control and stress conditions. Groups of rats were subjected to unilateral transections of the stria terminalis (ST), the medial forebrain bundle at the rostral hypothalamic level (RMFB), or the lower brainstem

through the medulla oblongata between the obex and the locus coeruleus (MH). Twelve days after surgery, each group of rats was further divided into controls (basal conditions) and stressed (1 hr immobilization), before collecting brains for mRNA analysis by in situ hybridization histochemistry. While ST and RMFB cuts had no effect on basal CRH mRNA levels in the PVN, MH decreased CRH mRNA in the PVN ipsilaterally to the knife cut but it was without effect on the contralateral side (-40% and -37% vs contralateral and sham operated, respectively, $p < 0.01$). Acute stress (rats were killed 3 hr after immobilization) increased CRH mRNA levels by about 30% bilaterally, an effect which was unchanged by ST, RMFB or MH. Under basal conditions CRH receptor mRNA levels were undistinguishable from the surrounding background in the PVN of sham operated controls, ST and RMFB operated rats. However, brain stem hemisection resulted in clear expression of CRH receptor mRNA in the dorsal, medial-ventral and lateral parvocellular subdivisions of the PVN, ipsilateral to the transection. CRH neurons in these subdivisions project to the lower brain stem and the spinal cord. Expression of CRH receptor mRNA in the medial-dorsal and anterior parvocellular divisions (CRH neurons with median eminence projections) was not affected by this MH cut. In these subdivisions, immobilization stress markedly increased CRH receptor mRNA levels but it did not influence MH cut-induced CRH receptor expression. ST and RMFB were without effect on PVN CRH receptor mRNA levels under basal or stress conditions. Oxytocin (OT) and vasopressin (VP) mRNA levels in the magnocellular subdivision of the PVN were unchanged after immobilization, or following ST, RMFB or MH cuts, whereas OT mRNA in the medial-ventral and caudal parvocellular subdivisions was decreased by 52% after MH cut. The data demonstrate that 1) basal CRH mRNA levels in the PVN are under tonic stimulatory influence of the lower brain stem (and/or spinal cord) afferents, 2) CRH receptor mRNA expression in PVN subdivisions (pituitary vs lower brainstem/spinal cord projecting neurons) is under different control mechanisms, and 3) immobilization stress induced changes in CRH mRNA and CRH receptor mRNA levels are mediated either by neural inputs from brain areas other than those investigated here, or by humoral factors.

The regulation of CRH receptor expression in the pituitary was further investigated by studying the effects of adrenalectomy and glucocorticoids on CRH binding and CRH receptor mRNA levels. CRH receptor mRNA levels decreased transiently following adrenalectomy (-62% after 18 hr), returning to basal levels after 4 or 6 day adrenalectomy, whereas binding of ^{125}I -Tyr-oCRH showed a sustained decrease. The early decrease was prevented by glucocorticoid replacement. Similar transient decreases in CRH receptor mRNA were observed by Northern blot analysis. In intact rats, dexamethasone (100 μg s.c.) caused a significant decrease in pituitary CRH receptor mRNA levels 2 to 10 hr after injection, returning to basal levels after 15 hr. On the other hand, dexamethasone (5 to 300 μg , s.c.) had no effect on pituitary CRH receptor mRNA levels 18 hr after injection. In the pituitary, changes in hypothalamic corticotropin releasing factors probably play a major role controlling CRH receptor mRNA levels during manipulations of circulating glucocorticoids levels. In addition, the inability of long term adrenalectomy and glucocorticoid administration to modify pituitary CRH receptor mRNA levels suggests that CRH receptor downregulation observed under these experimental conditions depends mainly on translational and post-translational events rather than receptor mRNA levels.

Significance to Biomedical Research and the Program of the Institute:

Exposure to different degrees of stressful stimuli is a prevalent problem in society. Stressful stimuli are integrated in the central nervous system and lead to a number of responses that include behavioral, visceral and hormonal adaptation. The changes in the hypothalamic-pituitary axis during prolonged stress can result in alteration of the secretion of most pituitary hormones with consequent alterations in metabolic, immune and reproductive function. Elucidation of the physiological mechanisms involved in the stress response will be important for the prevention and management of clinical conditions that involve disorders of neuroendocrine regulation.

Proposed Course:

Studies on the regulation of pituitary CRH and VP receptor expression will be extended to the molecular mechanisms. Initial studies will determine the second messengers involved in the regulation of the mRNAs of

these receptors in primary cultures of pituitary cells and in the cell line ATt-20. The cloning of the promoter region of the CRH receptor and VP receptor genes to study the transcriptional regulation of these receptors is a priority. A single genomic clone containing about 10kb upstream of the 5'untranslated region of the VP receptor has been obtained and is in the process of being sequenced. Studies are also in progress to determine the mechanisms involved in the dramatic changes in CRH receptor expression in the PVN. This include the role of glucocorticoids and afferent innervation of the PVN. The physiological role of CRH in regulating PVN function during stress is under study using CRH antagonists and antisense oligonucleotides to block the expression of the receptor.

Studies on the role of glucocorticoid feedback will be extended to the differential analysis of changes in glucocorticoid and mineralocorticoid receptors in pituitary and brain, as well as changes in 11 β -dehydrogenase, the enzyme which determines the interaction of glucocorticoids with the receptors. The expression of transcription factors which regulate the interaction of glucocorticoid receptors with glucocorticoid regulatory elements in the POMC gene will be studied in pituitary cells isolated from control and stressed rats and in cell lines. Studies will be continued to determine the role of catecholamines and neuropeptides in the expression and release of CRH and VP in the hypothalamus.

Publications

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Kiss A, Jezova D, Aguilera G. Activity of the hypothalamic pituitary adrenal axis and sympathoadrenal system during food and water deprivation in the rat, *Brain Res* 1994;663:84-92.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00632-06 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Physiological Actions of the Renin-Angiotensin-System

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	G. Aguilera	Head	SEP, DEB, NICHD
Others:	A. Kiss	Visiting Scientist	SEP, DEB, NICHD
	X. Luo	NRSA Fellow	SEP, DEB, NICHD

COOPERATING UNITS (if any)

None

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section Endocrine Physiology

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

0.7

PROFESSIONAL:

0.7

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to study physiological and pathological aspects of the renin-angiotensin system, emphasising its role in circulatory homeostasis and development. During the past year studies have focused on a) the role of type-1 angiotensin II (Ang II) receptors located in parvicellular corticotropin-releasing hormone CRH and CRH/VP cells in the hypothalamic paraventricular nucleus (PVN), and b) the consequences of chronic stress on the peripheral renin-Ang-aldosterone system.

a) The regulation of Ang II receptors in the PVN by glucocorticoids and the role of Ang II regulating the responsiveness of the HPA axis during stress was studied by correlating Ang II receptor expression in the PVN with changes in activity of the HPA axis. Various stress paradigms increased Ang II receptor expression in the parvicellular subdivision of the PVN in similar fashion irrespective of their effect on the activity of the HPA axis. Ang II receptor expression in parvicellular cells was reduced by adrenalectomy and increased by glucocorticoid administration. The stimulatory effect of stress on Ang II receptor expression in the PVN was prevented by adrenalectomy, indicating that glucocorticoids are necessary for the effect of stress. These data support a role for Ang II in the central mechanisms of adaptation to stress, but indicate that Ang II does not play a role controlling the responsiveness of the HPA axis.

b) Repeated physical-psychological stress reduced basal and stimulated aldosterone secretion in spite of elevated basal plasma renin activity. The increases in plasma renin activity were associated with increases in renin mRNA expression in the kidney due to repeated sympathetic stimulation. In vitro studies revealed that while the early aldosterone biosynthetic pathway is increased, Ang II receptor content, mRNA levels and activity of aldosterone synthetase are decreased in chronically stress rats. The data provides evidence for a role of chronic stress in the development of hyperreninemic hypoaldosteronism.

Project Description:Objectives:

The purpose of this project is to study the role of the renin-angiotensin-system in mammalian physiology with special emphasis on its role in the circulatory homeostasis and growth and development. Studies include characterization and mapping of the distribution of Ang II receptors subtypes and their function in the nervous system, endocrine organs and other peripheral tissues, and elucidation of the molecular mechanisms involved in the regulation of cellular function by the peptide. Studies on the control of mineralocorticoid secretion include analysis of the interaction of Ang II with other regulators, at the systemic and cellular level and to define the mechanisms responsible for changes in adrenal sensitivity to the peptide during changes in sodium intake.

Methods employed:

The majority of experiments are performed in the rat, and involve both in vivo and in vitro procedures. In vivo procedures include modifications of dietary electrolytes, administration of Ang II and inhibitors of the renin-ANG system and blood sampling in conscious rats with chronically implanted intravascular catheters. In vitro procedures include preparation of enzyme-dispersed fetal fibroblasts and adrenal glomerulosa cells, measurement of Ang II receptors in dispersed cells, membrane rich fractions and slide mounted tissue sections by conventional assays and by autoradiography; measurement of steroid production and intracellular messengers in isolated cells under basal and stimulated conditions; analysis of cellular proteins by gel electrophoresis and immunoblot techniques, and determination of mRNA by Northern blot, hybridization in solution and in situ hybridization.

Major findings:

Angiotensin II is a major regulator of adrenal mineralocorticoid secretion and blood pressure. In addition, components of the renin-angiotensin system are present in the brain and a number of peripheral tissues where it acts as a neurotransmitter or as a paracrine regulator. The effects of Ang II are mediated by plasma membrane receptors located in the adrenal zona glomerulosa and other target tissues. Two major subtypes of Ang II receptors have been identified, AT₁, which are coupled to calcium-phospholipid dependent signalling systems, and AT₂, with yet undefined actions. Previous studies of this laboratory have described the topographic localization and characterization of Ang II receptor subtypes in the brain and peripheral tissues.

Studies of this group using double staining in situ hybridization techniques have shown that Ang I type 1 receptors (AT₁) in the hypothalamic paraventricular nucleus (PVN) are located primarily in corticotropin releasing hormone (CRH) neurons of the parvicellular subdivision. Further studies were conducted to investigate the role of Ang II regulating the hypothalamic-pituitary adrenal (HPA) axis, by correlating AT₁ receptor expression levels in the PVN with the known changes in activity of the HPA axis under different stress paradigms, and manipulation of circulating glucocorticoids. AT₁ receptor mRNA was measured by in situ hybridization using ³⁵S-labelled cRNA probes and Ang II binding by autoradiography using ¹²⁵I[Sar¹,Ile⁸]Ang II in slide mounted hypothalamic sections. AT₁ receptor mRNA levels and Ang II binding in the PVN were reduced by about 20% 18 hr after adrenalectomy remaining at these levels up to 6 days thereafter. This effect was prevented by corticosterone administration in the drinking water, or dexamethasone injection (100 µg, s.c., daily). Conversely, dexamethasone injection in intact rats caused a 20% increase in AT₁ receptor mRNA in the PVN. AT₁ receptor mRNA and binding in the PVN increased 4 hr after exposure to stress paradigms associated with activation of the HPA axis (immobilization for one hr, or i.p. injection of 1.5 M NaCl), and remained elevated after repeated daily stress for 14 days. Unexpectedly, two osmotic stress models associated with inhibition of the HPA axis (60 hr water deprivation or 12 days of 2% saline intake) also resulted in increased AT₁ receptor mRNA levels and Ang II binding in the parvicellular PVN. In intact rats, the stimulatory effect of acute stress on AT₁ receptor mRNA in the PVN was significantly enhanced by dexamethasone administration (100 µg, s.c., 14 hr and 1 hr prior to stress), while in adrenalectomized rats, with or without glucocorticoid replacement, stress reduced rather than increased, AT₁ receptor mRNA. Dexamethasone, 100 µg, injected sc within 1 min the beginning of

immobilization in adrenalectomized rats, increased AT₁ receptor mRNA in the PVN to levels significantly higher than those after dexamethasone alone, indicating that the stress induced glucocorticoid surge is required for the stimulatory effect of stress on AT₁ receptor mRNA. The data suggests that AT₁ receptor expression in the PVN is under dual control during stress: stress-activated inhibitory pathways and the stimulatory effect of glucocorticoids. The lack of specificity of the changes in AT₁ receptor expression in the PVN following stressors with opposite effects on ACTH secretion (osmotic and physical-psychological stress) does not support a role for Ang II as a major determinant of the response of the HPA axis during stress.

Previous studies have shown that Ang II is the major regulator of aldosterone secretion. ACTH is also a potent stimulant in acute conditions but its prolonged administration markedly inhibits aldosterone secretion. Circulating levels of both Ang II and ACTH and therefore aldosterone increase in response to stress. Studies were conducted to determine the consequences of chronic stress on the renin-angiotensin-aldosterone system by analysis of plasma hormone levels, kidney renin mRNA levels, and adrenal Ang II receptors and steroidogenesis in rats under repeated immobilization (2 hr daily), or ip injection of 1.5 M NaCl (ipHS) for 14 days. 24 hr after the last stress in both stress models, plasma aldosterone levels were reduced in spite of significant increases in PRA. Repeatedly ipHS rats showed PRA responses to acute immobilization similar to controls, but markedly reduced plasma aldosterone responses. Concomitant with the increases in PRA, renin mRNA levels in the kidney were significantly increased in ipHS rats, and these increases were prevented by β -adrenergic receptor blockade with propranolol. In isolated adrenal glomerulosa cells from chronically stressed rats, maximum aldosterone responses to AII, ACTH and 8-Br-cAMP were significantly decreased, whereas pregnenolone responses were increased. P450-aldosterone synthetase mRNA levels and binding of ¹²⁵I[Sar¹,Ile⁸]AII were significantly reduced in the adrenal zona glomerulosa of stressed rats. These studies show that chronic repeated stress leads to renin stimulation due to sympathetic activation, and inhibition of aldosterone secretion due to inhibition of the late steroidogenic pathway. The data provides evidence for a role of chronic stress in the development of hyperreninemic hypoaldosteronism.

Significance to Biomedical Research and the Program of the Institute

The prominence of Ang II receptor expression during crucial periods of fetal and postnatal growth open new perspectives about the importance of the renin-AII system during development. Elucidation of the function of these novel receptors during development is of critical importance, especially in view of the increasing clinical use of drugs that affect the function of the renin-AII system. The studies on the central actions of Ang II are likely to uncover novel regulatory mechanisms in neuroendocrine function, including modulatory actions of the activity of the HPA axis, growth and reproductive function. In addition elucidation of the mechanism of regulation of adrenal glomerulosa cell function is relevant to understanding the pathogenesis and treatment of disorders involving alterations of mineralocorticoid secretion.

Proposed Course:

Future emphasis of this project will be placed in the elucidation of the neuroendocrine functions of AII. Based on the topographic localization of Ang II receptor mRNA in the PVN, the hypothesis to be tested is that Ang II has a modulatory effect on the function of the CRH neuron, and possibly on somatostatin and dopaminergic neurons of the PVN. Further in situ hybridization histochemistry studies will be performed to determine whether Ang II receptors are expressed in somatostatin and dopaminergic neurons in the PVN. The function of Ang II in the regulation of the HPA axis, growth hormone and prolactin secretion will be studied using Ang II receptor blockade with specific Ang II antagonists and antisense oligonucleotides during neuroendocrine manipulations, and by analysis of changes in Ang II receptor expression in relevant brain areas during manipulations of the HPA axis or gonadal hormones in male and female rats.

Publications

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00633-05 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Ovarian folliculogenesis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others:	Z.B. Tong	Visiting Fellow	SGR, DEB, NICHD
	J. Anasti	Clinical Associate	SGR, DEB, NICHD
	S. Nair	NRSA Fellow	SGR, DEB, NICHD
	R. Maity	Visiting Fellow	SGR, DEB, NICHD

COOPERATING UNITS (if any)

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 R. Caspi, Laboratory of Immunology NEI

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Gynecologic Research Section

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development

TOTAL STAFF YEARS:

3.0

PROFESSIONAL:

3.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project seeks to improve the clinical care available to patients with disorders of ovarian follicle function and ovulation through research using animal models and clinical protocols. In pursuing this goal, we expect to expand understanding of the ovarian follicle in health and disease. We have focused on premature ovarian failure, a condition that prematurely terminates ovarian function and fertility in 1% of women. During this period we found that femoral neck bone density is significantly reduced in women with premature ovarian failure. Surprisingly, more than one-half of our patients had a bone density low enough to cause a threefold increase their risk of fracture. Strikingly, we found luteinized graafian follicles in all the antral follicles we biopsied in 6 patients with karyotypically normal spontaneous premature ovarian failure. Therefore, luteinized graafian follicles account for at least 60% of the antral structures imaged (95% confidence limit). Inappropriate luteinization of graafian follicles appears to be a major pathophysiologic mechanism in patients with karyotypically normal spontaneous premature ovarian failure. We have particular interest in autoimmune ovarian failure. Using an animal model, we found that autoantibodies from mice with experimental autoimmune oophoritis bind to a 120 kd protein that is specific to the oocyte cytoplasm. By screening a mouse ovarian cDNA expression library with this serum, we identified a novel oocyte-specific gene that may represent the inciting antigen in this disease. Also, we showed that the inciting ovarian antigen in murine experimental autoimmune oophoritis appears to be produced independently of gonadotropin stimulation. By administering gonadotropin stimulation or suppression to neonatally thymectomized mice we did not alter the course of the disease. During this period we also found that the lymphocytic infiltration in murine experimental autoimmune oophoritis is primarily localized in the stroma and theca, and does not involve a direct lymphocytic attack against intact oocytes. Intriguingly, our investigations in murine post-thymectomy polyglandular autoimmunity suggest that neonatal thymectomy causes a deficiency of CD4+ T helper 1 (Th1) activity that persists into adulthood. In ongoing work we are testing the hypothesis that this Th1-deficient state induces the autoimmune disease. We will also determine, using transgenic technology, if the novel oocyte-specific gene is the inciting ovarian antigen in murine experimental autoimmune oophoritis. Future clinical directions involve developing strategies to increase bone density in women with premature ovarian failure, a treatment to prevent inappropriate luteinization in patients with premature ovarian failure, and a protocol to test the hypothesis that inappropriate luteinization plays a role in the infertility of maturing women.

Project DescriptionObjectives:

This project seeks to improve the clinical care available to patients with disorders of ovarian follicle function. We approach this goal from a broad perspective, aiming to advance knowledge of ovarian follicle physiology and pathophysiology through research using animal models of human reproductive disorders, and through clinical protocols evaluating diagnostic and therapeutic methods for these patients. The consolidating goal here is to develop methods to restore ovulation in patients currently unresponsive to available therapies. In the process of pursuing these goals, we expect to expand essential understanding of the ovarian follicle in health and disease. We now have specific interest in patients with premature ovarian failure. Approximately one percent of women experience ovarian failure prior to age 40. Although symptoms related to estrogen deficiency in these patients can be treated with estrogen replacement therapy, there is no known treatment to restore ovulatory function and make these women fertile. This problem has had greater impact in recent years. As modern women more commonly delay childbearing, more women will experience infertility as the most significant problem related to premature ovarian failure.

Our specific research objectives during the current period were directed along three main avenues.

1. Clinical research in premature ovarian failure

- a) Examine bone density in a group of these patients.
- b) Study the function of ovarian antral follicles in these women.
- c) Define the histopathology of these antral follicles to gain insight into the mechanism of ovarian follicle dysfunction.
- d) Evaluate treatments for patients with autoimmune oophoritis.

2. Basic research to develop clinical diagnostic markers for autoimmune premature ovarian failure

- a) Investigate circulating interfering immunoglobulins as a cause of premature ovarian failure.
- b) Explore antibodies against the zona pellucida as a marker for autoimmune premature ovarian failure.
- c) Examine the frequency of antibodies against human recombinant thyroid peroxidase and gastric parietal cells in patients with premature ovarian failure.

3. Basic research in murine post-thymectomy autoimmunity, an animal model of autoimmune premature ovarian failure

- a) Characterize the inciting ovarian antigen in this model.
- b) Define how neonatal thymectomy perturbs the developing immune system so as to induce autoimmunity.
- c) Evaluate the mouse major histocompatibility system (H2) as a determining factor in strain-specific susceptibility to post-thymectomy autoimmune oophoritis.

Methods Employed:Clinical premature ovarian failure

Premature ovarian failure is a condition causing amenorrhea, hypoenestrogenism, and elevated gonadotropins in women less than age 40. Patients with known abnormal karyotypes or evidence to suggest iatrogenic ovarian failure are not included in our studies.

Patients with premature ovarian failure are evaluated clinically to include: measurement of sex steroid hormones, gonadotropins, free T4, and thyroid stimulating hormone by radioimmunologic methods; karyotype analysis on cultured lymphocytes; Human Leucocyte Antigen (HLA) analysis by immunophenotyping using standard microcytotoxic typing with locally prepared HLA-DR1-10 antiserum trays; determination of bone density by dual energy x-ray absorptiometry; and evaluation of ovarian structure by ultrasonography. Patients may also undergo a three hour glucose tolerance test and ACTH stimulation test. Autoimmune activity is determined by immunohistochemistry, hemagglutination assay, and enzyme linked immunosorbent assay. Antiovarian antibodies in women are detected by indirect immunofluorescence using M. cynomolgus ovary as tissue substrate. These clinical investigations involve the participation of either healthy human subjects or patients.

Previous investigators have used ovarian wedge biopsy in the evaluation of premature ovarian failure. However, wedge biopsy was originally developed as a technique used in polycystic ovarian syndrome, a condition associated with abnormally large ovaries. Patients with premature ovarian failure have small ovaries, and wedge biopsy in this condition has tremendous sampling error. Therefore, rather than performing wedge biopsy at a random time, we have performed directed ovarian follicle biopsy when an ovarian antral follicle was visualized by pelvic ultrasound. Ovarian biopsy is performed under general anesthesia by minilaparotomy or laparoscopy. Biopsy specimens are processed and stained routinely with hematoxylin and eosin, and immunohistochemical studies are performed to detect lymphocytic infiltration.

We test for interfering antibodies in women with premature ovarian failure using a recombinant system expressing human FSH and LH receptors. A mouse adrenal cell line transfected with the human FSH receptor gene (Y1-hFSHR) exhibits a dose dependent increase in progesterone when exposed to hFSH. An embryonal kidney cell line transfected with the human LH receptor gene (hLHR-293) exhibits a dose dependent increase in cAMP when exposed to hLH. We isolated immunoglobulins from patients with premature ovarian failure and normal women. Anti-gonadotropin polyclonal antibodies isolated in the same manner were used as a positive control. We stimulated Y1-hFSHR and hLHR-293 cells with hFSH or hLH in the presence of these immunoglobulins and determined the resulting progesterone or cAMP response.

Murine post-thymectomy polyglandular autoimmunity

Thymectomy performed two to four days after birth induces murine polyglandular autoimmunity in a strain-specific manner. Mice may develop autoimmune thyroiditis, autoimmune gastritis, or autoimmune oophoritis, a constellation of autoimmunity that is seen in the clinic. We work primarily with (C57/BL6 x A/J)F1 hybrids (B6A) because 90% of B6A mice develop experimental autoimmune oophoritis after neonatal thymectomy.

To characterize the autoantibodies in murine experimental oophoritis we use immunoblotting. Ovarian proteins are prepared for electrophoresis on SDS-PAGE gel, transferred to nitrocellulose membrane, incubated with sera from thymectomized or control mice, and developed with secondary antibody using immunoperoxidase. To identify the ovarian proteins that bind antibody, we screen a mouse ovarian cDNA library with serum from mice with high-titer ovarian autoantibodies.

Previous cell transfer studies, using cells from spleens of normal adult mice, have shown that mice thymectomized as neonates are deficient in a CD4+ T cell population that can prevent autoimmunity. We seek to determine if this is a deficiency in CD4+ T helper 1 (TH1) or T helper 2 (TH2) activity. To characterize CD4+ TH1 and TH2

activity, we stimulate CD4⁺ splenic lymphocytes in vitro by engaging CD3 and CD28 with specific antibodies. We measure interferon-gamma (INF-gamma) production, a measure of CD4⁺ TH1 cell activity, by enzyme-linked immunosorbent assay (ELISA). We measure interleukin-4 (IL-4) production, a measure of TH2 cell activity, by utilizing a proliferation assay employing an IL-4-dependent cell line. We enrich for CD4⁺ T cells by magnetic separation and confirm the separation by flow cytometry. To characterize the location of the inciting ovarian antigen in this model we stain ovaries with immunoperoxidase immunohistochemistry using monoclonal antibodies to CD45, a cell surface protein specific to mouse leukocytes. We evaluate the importance of the mouse major histocompatibility system by performing neonatal thymectomy on congenic strains of mice that differ only at the H2 locus.

Major Findings:

1. Clinical premature ovarian failure

a. Bone density

We found that, based on bone density measurements, over one-half of our patients with premature ovarian failure have a threefold increased risk of hip fracture. This is despite the fact that 85% of these patients had been taking estrogen replacement therapy. We found that 48 of 89 women with premature ovarian failure (54%) had a bone density at the femoral neck less than one standard deviation below the mean of normal age-matched women ($p < 0.001$, chi square goodness of fit test). A bone density less than one standard deviation below the mean is associated with a threefold increase in the risk of fracture.

b. Antral follicle function

At one time it was assumed that premature ovarian failure was caused by depletion of ovarian follicles, and, like menopause, is an irreversible condition. We continue to collect evidence to refute this view.

Many patients with karyotypically normal spontaneous premature ovarian failure appear to have a store of primordial follicles that are appropriately "timed" to become activated over an extended period, but the redundancy in follicle number needed to assure the constant availability of a cohort of developing follicles is deficient. Women require the constant availability of a cohort of developing follicles to maintain regular ovulatory menstrual cycles.

We detected ovarian follicle function in approximately 50% of patients with karyotypically normal spontaneous premature ovarian failure by measuring serial estradiol blood levels over 4 months. Furthermore, we imaged an antral follicle by sonography in over 40% (27/65) of our patients with premature ovarian failure. The median ovarian follicle size was 8 mm (range 3 to 46). Interestingly, the probability of detecting a follicle did not appear to decline as the time from diagnosis increased. Even patients more than six years since diagnosis had antral follicles detected. We know from previous work that follicles are few and far between in ovarian biopsies taken from patients with karyotypically normal spontaneous premature ovarian failure.

The antral follicles in these patients are endocrinologically active, and not mere atretic remnants of follicles. Serum estradiol was significantly greater when an antral follicle was present ($p < 0.01$), and this is evidence that these structures are indeed endocrinologically active. But the follicles in these patients were not functioning normally. We could detect only a weak trend toward a positive correlation between serum estradiol levels and follicle diameter in women with karyotypically normal spontaneous premature ovarian failure ($r = 0.24$, $p < 0.10$). By contrast, in normal women serum estradiol levels have a strong positive correlation with follicle diameter ($r = 0.91$, $p < 0.005$). During the follicular phase in normal women serum progesterone levels are also strongly correlated with follicle diameter ($r = 0.83$, $p < 0.005$). We did not detect a correlation between serum progesterone levels and follicle diameter in patients with premature ovarian failure.

With this evidence of abnormal follicle function, we biopsied ovarian follicles in a few patients under an approved protocol to gain insight into the mechanism causing dysfunction. We sought to determine if autoimmune oophoritis was impairing follicle function or was this some other mechanism?

c. Antral follicle histology

Strikingly, we found luteinized graafian follicles in all the antral follicles we biopsied in 6 patients with karyotypically normal spontaneous premature ovarian failure. Therefore, luteinized graafian follicles account for at least 60% of the antral structures imaged (95% confidence limit). Inappropriate luteinization of graafian follicles appears to be a major pathophysiologic mechanism in patients with karyotypically normal spontaneous premature ovarian failure.

In these patients, apparently due to an inadequate cohort of follicles, LH fails to be suppressed into the normal range, and this leads to inappropriate luteinization of the few graafian follicles capable of responding to FSH stimulation. A properly timed LH surge luteinizes a mature follicle, reduces granulosa cell mitogenic activity, and induces changes in the steady state level of mRNAs for enzymes involved in progesterone production. Inappropriate luteinization of a growing follicle would thus be expected to impair follicle growth and reduce estradiol production. The elevation of serum LH during the normal menopausal transition is thought to result from inadequate negative feedback due to the reduced number of follicles. In a normal menstrual cycle, the cohort of atresia-destined follicles may play a more important role than has been recognized. This cohort maintains a proper gonadotropin environment and thus prevents premature luteinization.

d. Treatment of autoimmune premature ovarian failure

Two previous studies, one by our laboratory and one by a laboratory in Boston, have demonstrated that patients with premature ovarian failure as a group have increased T lymphocyte activation in peripheral blood. This suggests autoimmunity plays a role in a substantial portion of these patients. However, we have no serum marker to accurately identify individual women who have premature ovarian failure on an autoimmune basis.

As part of a controlled study evaluating alternate-day prednisone therapy for patients with biopsy-proven autoimmune oophoritis, we biopsied developing antral ovarian follicles in six women. Despite our use of sensitive immunohistochemical methods, we did not find autoimmune oophoritis in any ovarian follicle biopsies performed on these patients. We have, therefore, modified our patient selection criteria to include patients with other evidence to suggest autoimmunity. Also, based on our findings in the mouse model, we suspect the disease can sometimes be sequestered in the ovarian hilum. Therefore, we have modified our ovarian biopsy technique. We are currently evaluating a novel laparoscopic ovarian biopsy strategy that will be certain to obtain a portion of the ovarian hilum.

During this period we completed a prospective, double blind placebo-controlled trial evaluating danazol treatment for patients with premature ovarian failure. We tested the hypothesis that a period of ovarian rest might reduce antigenic load and allow ovarian function to return after the suppression is withdrawn. Danazol is a weak androgen that suppresses gonadotropins and has known favorable immunomodulatory effects. Danazol, did not improve ovarian function.

2. Diagnostic markers for autoimmune ovarian failure

a. Interfering antibodies

Circulating immunoglobulins that interfere with the interaction of FSH and LH on their respective receptors appear to be an uncommon cause of premature ovarian failure. Despite using human receptors and human gonadotropins, we did not detect circulating antibodies inhibiting this interaction in any of 38 patients with premature ovarian failure. Therefore, if blocking antibodies that interfere with gonadotropin-receptor interaction

are a mechanism for premature ovarian failure, they account for a minority of cases (<8%, 95% confidence limit).

b. Zona pellucida antibodies

Strikingly, we found that approximately one-third of normal women (n=26) had these antibodies. Thus, the test is not helpful to women with premature ovarian failure. However, because one-half of our patients with premature ovarian failure had these antibodies, we attempted to improve the specificity of the test. We could not improve the test by using higher serum dilutions or simplified outcome measures.

We are encouraged, however, by that fact that we did not detect zona pellucida antibodies in any of 26 men ($p < 0.001$). This is intriguing. This finding supports recent proposals that pre-B cells undergo positive selection directed by the presence of surface heavy chain with low affinity to autoantigen. It appears then, that men, lacking this self antigen, fail to provide positive selection for these pre-B cells clones. Also, this finding suggests that our detection system is specific for zona pellucida, and that further refinement of the assay system by using pure human recombinant zona pellucida protein might be useful.

c. Thyroid peroxidase and gastric parietal cell antibodies

We found that women with karyotypically normal spontaneous premature ovarian failure have a significantly higher incidence of antibodies against human thyroid peroxidase and gastric parietal cells as compared to age-matched normal women. The constellation of autoimmunity seen in murine post-thymectomy autoimmune oophoritis includes autoimmunity against the thyroid and gastric parietal cells. Therefore, we determined whether a similar constellation of autoimmunity was present in patients with premature ovarian failure. Our finding suggests this mouse model may indeed have expression in human pathology, and studies to define the inciting antigen in this mouse model may have important implications to women with premature ovarian failure.

Murine post-thymectomy polyglandular autoimmunity

a. Inciting ovarian antigen

We have identified a novel oocyte-specific gene. Previously, we found that autoantibodies from mice with autoimmune oophoritis bind to a 120 kd protein that is specific to the oocyte cytoplasm. We identified the novel gene by screening a mouse ovarian cDNA expression library with this serum. We have demonstrated that the message is specific to the ovary by RNase protection assay, and we have demonstrated that the message is specific to the oocyte by in situ hybridization. These mice not only develop high-titer autoantibodies specific to oocytes, but ultimately the ovaries become fibrotic and devoid of primordial follicles. These findings implicate the oocyte as a primary target of the autoimmune process.

We have shown that the inciting antigen in murine experimental autoimmune oophoritis appears to be produced independently of gonadotropin stimulation of the ovary. These findings are consistent with the hypothesis that the inciting antigen is present in oocytes of primary follicles, as suggested by the oocyte specificity of the high-titer serum. Primary follicle development does not require gonadotropin stimulation. By administering gonadotropin stimulation to neonatally thymectomized mice we did not exacerbate existing disease, or induce an earlier onset of severe disease. Furthermore, by administering gonadotropin suppression to neonatally thymectomized mice we did not reduce the degree of lymphocytic infiltration or oocyte destruction.

During this period we found that even the anamnestic development of lymphocytic infiltration in murine experimental autoimmune oophoritis was primarily localized in the stroma and theca. On the surface, at least, this appears inconsistent with the hypothesis that the inciting antigen is an oocyte protein. In previous work we demonstrated that in developing murine autoimmune oophoritis the lymphocytic infiltration was confined to the stroma and theca, and not found involving oocytes. Therefore, we investigated the possibility that lymphocytic infiltration involving oocytes develops as part of end-stage disease. We transplanted normal syngeneic ovaries

to B6A mice with confirmed autoimmune ovarian failure, and, as a control, to normal oophorectomized mice. Our findings suggest that the ovarian failure in this model is not mediated by a direct lymphocytic attack against intact oocytes. Other immune-mediated mechanisms are responsible. The paradoxical development of high-titer oocyte-specific antibodies despite the stromal and thecal location of the lymphocytic infiltration remains to be explained.

b. Perturbing the neonatal immune system

Adoptive transfer of CD4+ splenic lymphocytes from normal adult mice prevents the disease from developing in neonatally thymectomized mice. The mechanisms of this regulation, and the reason why the timing of neonatal thymectomy is so critical, are unclear.

We have findings to suggest that 1) neonatal thymectomy induces autoimmunity by impairing development of T helper type 1 (Th1) regulation, and 2) the postnatal shift to normal adult Th1/Th2 balance is established by a thymus-dependent process. These results hold implications for the pathogenesis, and possibly for the therapy of autoimmune polyglandular failure in humans.

Strikingly, we found that splenic CD4+ cells from adult mice thymectomized as neonates produced an inappropriate neonatal-like Th2 predominant response (high levels of IL-4 and low levels of interferon gamma). Cells from adult mice sham-operated as neonates produced the expected Th1 predominant response seen normally in adult mice (low levels of IL-4 and high levels of interferon gamma).

We also found that administration of interleukin-12, a key cytokine that promotes CD4+ T cell differentiation towards the Th1 phenotype, restored the adult-like Th1 pattern of mice thymectomized as neonates, and ameliorated the development of autoimmune oophoritis. We found that postnatal adoptive transfer of adult splenocytes to mice thymectomized as neonates had the same effect.

During the current period we also showed that mice thymectomized as neonates have reduced natural killer (NK) cell activity. IL-12 also stimulates NK cells, and increases their production of INF-gamma. This is an additional indirect mechanism by which IL-12 promotes CD4+ cell differentiation toward Th1. IL-12, administered after neonatal thymectomy, appears to in some way compensate for the lack of thymic function. This raises the possibility that the thymus effects the change in the periphery by secretion of IL-12. The message for both the p35 and p40 chains of IL-12 are produced in mouse thymus.

c. Major histocompatibility complex

During the current period, by using congenic strains of mice in a well-controlled experiment, we showed that genetic background plays a more important role than H-2 haplotype in the development of post-thymectomy autoimmune oophoritis. The availability of congenic strains of mice makes it possible to separate the effects of the genetic background and the specific H-2 major histocompatibility complex. "A" mice (A/J) are highly susceptible to post thymectomy autoimmunity, whereas "B" mice (C57 BL10) are relatively resistant. We showed that A.By mice developed severe autoimmune oophoritis equivalent to A/J mice. A.By mice have the "A" genetic background, but the "b" H-2 major histocompatibility complex. Therefore, severe disease can develop in "A" mice despite the absence of the "a" H-2. Furthermore, we found that B10.A mice, which have the "B" background and the "a" H-2, did not develop disease. Therefore, the presence of the "a" H-2 alone is insufficient to convey disease susceptibility.

Significance to Biomedical Research and the Program of the Institute:Clinical premature ovarian failurea. Bone density

It is striking that, based on bone density at the femoral neck, there is a threefold increase in risk of hip fracture in over one-half of our patients with premature ovarian failure. Hip fracture is associated with major morbidity. The dollar cost of hip fracture in the United States alone is more than \$8 billion per year. In addition, there are human costs of pain, functional limitation, reduced quality of life, loss of independence, and inability to work. There is evidence that American women in general are experiencing more hip fractures earlier in life. In other words, the age-specific incidence of hip fracture has been increasing during the past three decades. The reasons for this age-specific increase are unknown, but increasingly sedentary life styles likely play a role.

Even simple questions with tremendous public health import about osteoporosis go unanswered. For example, presently we do not know what type of weight bearing exercise is the most effective to increase bone density in young women. Our patients with premature ovarian failure are an ideal group in which to evaluate strategies to increase bone density and reduce risk of hip fracture. Patients with premature ovarian failure experience periods of hypoestrogenism before the disorder is recognized, and hypoestrogenism causes rapid loss of bone mineral density. Estrogen replacement therapy will halt this rapid loss, but alone will not restore lost bone. A safe and effective method to restore bone density for these women is needed. One in 100 American women develop premature ovarian failure.

b. Luteinized graafian follicles

By identifying luteinization of graafian follicles as a major pathophysiologic mechanism in patients with premature ovarian failure, this program has provided key insight into ovarian follicle function in these patients. When viewed from a broader perspective, this finding has public health implications to human reproduction that loom large and demand further investigation. Development of luteinized graafian follicles in patients with premature ovarian failure may be only one extreme of a vast continuum of human reproductive pathophysiology.

Our findings raise the distinct possibility that reproductive pathology in maturing women may result from the decline in primordial follicle number. Reduced negative feedback on LH rather than an intrinsic defect within the individual remaining primordial follicles may be the mechanism of fertility decline. With advancing age, along with this decline in follicle number, women experience a concomitant dramatic decline in fertility, increase in the rate of pregnancy wastage, and increase in births resulting from chromosomal nondisjunction (trisomy 21). This reproductive pathology has been traditionally ascribed to "ovarian aging," but the pathophysiologic mechanism of this deterioration in reproductive function has never been defined.

Our findings suggest that the excess supply of primordial follicles present in normal women plays a critical role by providing a cohort of follicles to provide negative feedback. This negative feedback maintains LH in the normal range, and prevents inappropriate luteinization of developing graafian follicles. Women begin puberty with approximately 300,000 primordial follicles in their ovaries, but only about 400 of these follicles actually ovulate during a woman's reproductive life. Whether there is a physiologic need for this vast excess of primordial follicles has been a mystery.

Subtly inadequate negative feedback upon LH, subtle enough to evade detection by available clinical measures, might impair normal dominant follicle function and egg maturation in older menstruating women. If true, strategies to restore normal negative feedback on LH might improve fertility, reduce pregnancy wastage, and possibly even improve oocyte function to reduce the incidence of chromosomal nondisjunction.

c. Autoimmune oophoritis

Our findings highlight the fallacy of adding treatments into clinical practice based on anecdotal reports of success, and emphasize the need for controlled studies to evaluate therapies for this condition. Patients with premature ovarian failure often experience spontaneous remission.

Anecdotal reports have suggested that high-dose, long-term prednisone therapy may be useful in treating autoimmune ovarian failure. However, prednisone, when used in high-dose for a long-term, has substantial side effects. These include aseptic necrosis of bone requiring major surgical intervention. Despite this risk, patients with premature ovarian failure are being treated based on this anecdotal evidence. We are aware of one patient with premature ovarian failure who developed aseptic necrosis of bone on high-dose, long-term prednisone therapy administered elsewhere.

To make progress toward a better understanding of human autoimmune oophoritis will require an integrated approach, such as presented here, to combine clinical and basic laboratory investigation with use of appropriate animal models. A major obstacle to progress in autoimmune premature ovarian failure is the lack of an accurate diagnostic method to identify patients who develop ovarian failure by this mechanism. The need is great to define the pathophysiology of autoimmune ovarian failure, to develop accurate tests to identify the pathogenic mechanisms, and to develop specific efficient therapies to reverse these pathogenic mechanisms without the side effects that accompany generalized immunosuppression.

We are narrowing the search for autoimmune oophoritis. Work by previous investigators has demonstrated that ovarian wedge biopsy has a low yield in diagnosing autoimmune oophoritis. Based on our findings it appears that ovarian biopsy targeted at developing antral follicles will also have low yield in diagnosing this condition.

As practicing physicians become aware of our findings through peer review publications and book chapters, unnecessary health care expenditures related to premature ovarian failure should decline, and these funds will become available for other health care needs. Clinicians seeing patients with premature ovarian failure often order antiovarian antibody titers in an effort to diagnose autoimmune premature ovarian failure. Some also order lymphocyte phenotyping to determine if HLA DR3 is present. These tests are expensive, and, based on our findings, add nothing substantive to the evaluation.

Murine post-thymectomy polyglandular autoimmunity

Autoimmune diseases rank among the major medical problems of today's industrialized societies. Women are more susceptible to autoimmune diseases than men, and autoimmune diseases in children can significantly impair normal growth and development. Independent from their medical and social consequences, autoimmune diseases present fundamental questions about the development and regulation of the immune system. What mechanisms permit the immune system to differentiate autoantigen from invading antigen? Murine post-thymectomy polyglandular autoimmunity is particularly appealing as a model to gain insight into this development and regulation. In this model, autoimmunity is induced by a specific perturbation to the immune system, without the need for administration of exogenous antigen.

a. Inciting ovarian antigen

The novel oocyte-specific gene we have identified just might be the gene that produces the inciting ovarian antigen in this murine model. This gene may allow us to develop a sensitive and specific serum marker for human autoimmune ovarian failure. If we detect an analogous human gene, we can employ the analogous human recombinant protein in an assay to detect oocyte-specific human ovarian autoantibodies. There is precedence for this strategy. In murine post-thymectomy autoimmune gastritis the inciting antigen has been identified as the gastric proton pump. Interestingly, antibodies to the gastric proton pump are also a useful marker for autoimmune gastritis in humans. In many models of organ-specific autoimmunity a single protein antigen plays a key role

in initiating disease, and dramatic cellular and humoral responses to this antigen develop early in the process.

b) Perturbing the neonatal immune system

We may have uncovered the major pathophysiologic mechanism by which neonatal thymectomy induces polyglandular autoimmunity. Our findings challenge fundamental mechanisms that have been proposed regarding the development of the neonatal immune system. Furthermore, our findings provide a basis for evaluating and treating human autoimmune disorders from the perspective of abnormal neonatal development.

Our studies in this model might prove basis for a clinical trial of IL-12 therapy for children with the candidiasis endocrinopathy syndrome (autoimmune polyglandular failure type 1). This is an enigmatic autosomal recessive syndrome in which children with impaired cellular immunity, a TH1 dependent function, nevertheless develop autoimmune polyglandular failure.

Proposed Course:

Basic research

We will finish sequencing the novel murine oocyte-specific gene that we identified. To identify this gene, we screened a mouse ovarian lambda gt-11 cDNA expression library with high-titer serum from mice with post-thymectomy autoimmune oophoritis. If we can sequence an analogous human gene, we will employ the analogous human recombinant protein to develop an assay for high-titer oocyte-specific autoantibodies in patients with premature ovarian failure. We will also evaluate the use of human recombinant zona pellucida 3 (ZP3) protein as a more specific test to determine if patients with premature ovarian failure have antibodies against the human zona pellucida (radio-immunoprecipitation assay).

We will determine if our novel oocyte-specific gene produces the inciting antigen in murine post-thymectomy autoimmune oophoritis. We will do this by expressing this single autoantigen in the thymus using transgenic techniques. If this is indeed the inciting antigen, the expression of this protein in the thymus should induce specific tolerance. This should abrogate the development of autoimmune oophoritis in A/J mice thymectomized as neonates. This same approach has been used previously to confirm that one specific protein, the β subunit of H/K ATPase (gastric proton pump), is the inciting antigen in post-thymectomy autoimmune gastritis.

We will extend our experiments in murine post-thymectomy polyglandular autoimmunity to further study the development and regulation of the immune system. If neonatal thymectomy induces disease by impairing the development of a Th1 response, disease should not occur if we can cause the shift to the adult Th1 dominant response to occur before the thymectomy is performed on day 4 of life. To test this hypothesis, we will administer IL-12 on day 2 and 3 of life, perform thymectomy on day 4 as usual, then determine if this treatment restores normal Th1:Th2 balance and ameliorates the autoimmune oophoritis despite thymectomy.

We will also extend our experiments into the clinic by examining children with the candidiasis-endocrinopathy syndrome (autoimmune polyglandular failure type 1) for evidence of a similarly predominant TH2 response in peripheral blood lymphocytes. If in these children such an abnormality in the normal TH1:TH2 profile is indeed present, this would raise the possibility of a subsequent clinical trial employing IL-12 as a means not only to treat the chronic candidiasis, but also to possibly prevent the development of polyglandular failure.

Clinical research

We will continue our controlled trial of alternate day prednisone therapy in the treatment of biopsy-proven autoimmune premature ovarian failure. This protocol tests the hypothesis that a lower risk therapy (alternate-day, lower dose, shorter-term prednisone) will induce remission of autoimmune ovarian failure.

We will begin a randomized controlled trial to evaluate strategies to improve bone density in women with premature ovarian failure. We will compare programs of intensive home-based weight-bearing exercise specifically designed to maximally load the hips and spine. Change in bone mineral density at the femoral neck measured by dual energy x-ray absorptiometry will be the primary outcome parameter to be compared.

We will extend our investigation of luteinization of graafian follicles in patients with premature ovarian failure. We hypothesize that by suppressing gonadotropins, and then stimulating follicle growth with human recombinant FSH, we may prevent premature luteinization of follicles and improve ovulation rates. By using an open trial design, this study will test the hypothesis that this treatment induces ovulation in 33% of patients. We learned in previous studies that women with premature ovarian failure, who have a serum estradiol greater than 50 pg/ml during one two month period of weekly sampling, have an 8.7% chance of having an ovulatory serum progesterone level during a second independent two month period of observation (upper 95% confidence limit 32%).

We will extend our clinical investigation to determine if luteinized graafian follicles may represent one extreme of a continuum of human reproductive pathophysiology. No therapy proven by prospective controlled study is available to women over 40 years old who have unexplained infertility. We hypothesize that in women over 40 who have unexplained infertility, by suppressing serum LH and then stimulating follicle growth and development with human recombinant FSH, we may prevent subtle premature luteinization of follicles and improve pregnancy rates. This study will test this hypothesis by a randomized, double-blind, placebo controlled, crossover design study. We will use the luteinizing hormone releasing hormone analog D-Trp⁶ GnRH (D-Trp⁶-Pro⁹-NEt-GnRH) to suppress serum LH, and recombinant human FSH to stimulate follicle growth. Women with three years of unexplained infertility have a per cycle fecundity of 0.01. This treatment would be a practical therapy if it could increase the per cycle fecundity to 0.15. We need to enroll 95 patients to detect this difference 90% of the time.

We will investigate vascular endothelial growth factor as an early molecular marker for ovarian follicle luteinization. To develop new therapies for patients with abnormal ovarian follicle function requires an understanding of normal human ovarian function. Surprisingly, little is known about the local factors that effect and regulate human follicle luteinization. Inappropriate luteinization of graafian follicles could be a major pathophysiologic mechanism preventing normal follicle function in many reproductive disorders. Neovascularization is a major histologic component of luteinization, and therefore, vascular endothelial growth factor might be a useful early molecular marker. Thirty healthy women who will be undergoing reversal of surgical sterilization by minilaparotomy will be recruited. They will have their surgery timed to take place within a 4 day window expected to include the luteinizing hormone (LH) surge. The time of the surge will be determined from blood samples collected every 4 hours during the days preceding surgery. At the time of tubal surgery, the dominant ovarian follicle will be excised using microsurgical techniques. The follicle will be snap frozen, sectioned, and prepared with appropriate radio-labelled probes to detect message for vascular endothelial growth factor. Signal intensity in the theca and granulosa cells will be compared in follicles removed before and after the LH surge. We will also continue our protocol that permits us to aspirate granulosa cells, follicular fluid, and oocytes from the follicles of women undergoing sterilization.

We will investigate a method of ovulation induction for patients with Stein Leventhal syndrome who are resistant to clomiphene citrate therapy. Ovarian follicles contain androgen receptors, and androgens may play a role in follicle atresia. The hyperandrogenism associated with Stein Leventhal syndrome might impair ovarian follicle development through a receptor-mediated mechanism. Flutamide, a non-steroidal anti-androgen, effectively blocks the action of androgen at the receptor level. We will test the hypothesis that blockade of androgen action with flutamide will permit ovulation in women previously resistant to clomiphene citrate alone. We will employ a randomized, double blind, and placebo controlled study design. We will study 50 patients and determine ovulation rates by serial progesterone levels.

In a new area of investigation for this project, we will evaluate fat-suppressed magnetic resonance imaging (MRI) as a non-invasive method to detect lesions of endometriosis in patients with severe invasive endometriosis.

Endometriosis is associated with infertility in women, and evidence suggests the presence of endometriosis may interfere with normal ovarian follicle growth and development. While not classified as a malignancy, severe invasive endometriosis can nevertheless invade bowel, bladder, and even lead to ureteral obstruction. Often the disorder involves prolonged medical treatment and repeated surgical interventions. All currently available conservative treatment strategies for endometriosis are, in essence, palliative. An accurate, non-invasive method to detect and monitor endometriosis would facilitate efforts to develop more effective conservative therapies. Twenty patients with severe invasive endometriosis will be recruited. After appropriate preoperative evaluation and MRI examination, patients will undergo surgery as indicated by their individual clinical situation. The size and location of endometrial implants in the surgical field will be noted. The sensitivity, specificity, and positive predictive value for the fat-saturated MRI technique to detect 4 mm lesions will be generated by standard statistical methods.

Protocols:

91-CH-127 Nelson Ovarian follicle function in patients with premature ovarian failure

This screening protocol is the focal point of our clinical and basic research effort on premature ovarian failure. We use this enigmatic disorder as a springboard to gain insight into ovarian function not only by seeing patients, but also by employing animal models and basic molecular biology in collaboration with basic scientists. In so doing, we take full advantage of the unique environment provided by the NIH Clinical Center. The potential for a broad public health impact of such an approach should not be underestimated. We have recruited 51 of the 150 patients approved for this protocol. From this protocol we have learned that one-half of patients with premature ovarian failure have follicles that function intermittently, and based on controlled studies, we have also found that patients with this disorder can undergo remission after treatment with placebo. This emphasizes the need for controlled studies to evaluate treatments for this disorder.

92-CH-223 Nelson Autoimmune premature ovarian failure: a controlled trial of alternate-day prednisone therapy

No therapy for infertile patients with premature ovarian failure has been proven effective by prospective controlled study. Anecdotal reports have suggested that high-dose, long-term prednisone therapy may be useful in treating autoimmune ovarian failure. However, prednisone, when used in high-dose for a long-term has substantial side effects, including aseptic necrosis of bone requiring major surgical intervention. Despite this risk, patients with premature ovarian failure are being treated based on this anecdotal evidence. We are aware of one patient with premature ovarian failure who developed aseptic necrosis of bone on high-dose, long-term prednisone therapy administered elsewhere. This protocol will test the hypothesis that a lower risk therapy (alternate-day, lower dose, shorter-term prednisone) will induce remission of autoimmune ovarian failure. The protocol will use a double-masked, placebo-controlled design. Patients will undergo ovarian biopsy by laparoscopy to confirm the presence of autoimmune oophoritis. Ovarian biopsy carries a mortality risk approximately equivalent to the risk women accept when they decide to undergo surgical sterilization. Successful outcome will be defined as a return of ovulation as determined by weekly serum progesterone levels. The hypothesis that short-term, alternate-day prednisone therapy restores ovulation will be tested with an equality of proportions test comparing the proportion of patients who ovulate during placebo with the proportion of patients who ovulate during prednisone therapy.

92-CH-153 Anasti Aspiration of granulosa cells, follicular fluid, and oocytes in women undergoing sterilization

To develop new therapies for patients with abnormal ovarian follicle function requires an understanding of normal human ovarian function. This protocol permits us to collect human ovarian follicle fluid, human granulosa cells, and human oocytes. This enables us to define the normal biochemical and cellular parameters related to normal ovarian follicle function and ovum maturation. We obtain these materials from women undergoing laparoscopic sterilization. To be eligible for this protocol, women must be referred to us by their personal physician after they

have independently decided to undergo laparoscopic sterilization. Aspiration of ovarian follicles during laparoscopic sterilization has negligible risk. As part of protocol 92-CH-223 (above) we have demonstrated that patients with premature ovarian failure develop graafian follicles that are inappropriately luteinized. We have moved ahead under this protocol to investigate the process of luteinization in normal women. We have shown that human follicle fluid TGF β (transforming growth factor β) is positively correlated with follicle fluid progesterone levels, a marker for luteinization. This suggests TGF β may have a role in human granulosa cell luteinization.

92-CH-16 Anasti Flutamide in the treatment of Stein-Leventhal Syndrome

Anovulation associated with Stein Leventhal Syndrome causes infertility. Clomiphene citrate therapy is the first line of treatment for women with this condition who desire fertility. However, in some patients clomiphene citrate fails to induce ovulation. Presently, we have no effective therapy for patients with who fail to respond to clomiphene. Ovarian follicles contain androgen receptors, and the hyperandrogenism associated with Stein Leventhal Syndrome might impair follicular development through a receptor-mediated mechanism. Flutamide, a non-steroidal anti-androgen, effectively blocks the action of androgen at the receptor level. This protocol tests the hypothesis that blockade of androgen action with flutamide will allow patients to ovulate even though they were previously resistant to clomiphene therapy alone. We employ a randomized, double blind, and placebo controlled study design. We plan to study 50 patients and determine ovulation rates by serial progesterone levels. Recruitment has been slow, and we plan to step up the effort on this protocol during the coming year.

93-039 Nair Immunogenetics of autoimmune oophoritis in the neonatally thymectomized mouse model

93-032 Nelson Autoimmune ovarian failure (murine)

Publications

Anasti JN, Flack MR, Froehlich J, Nelson LM. The use of human recombinant gonadotropin receptors to search for IgG mediated premature ovarian failure, *J Clin Endocrinol Metab* 1995;80:824-8.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00634-03 DEB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Metabolic Effects of Insulin-like Growth Factor I in Normal and Diabetic Adolescents.

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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Others: Mark Bach Senior Staff Fellow SGM, DEB, NICHD
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COOPERATING UNITS (if any)

None

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Growth and Metabolism

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00636-03 DEB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Endocrinology of Reproduction in Women

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LAB/BRANCH

Developmental Endocrinology Branch

SECTION

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INSTITUTE AND LOCATION

National Institute of Child Health and Human Development, NIH, Bethesda, MD

TOTAL STAFF YEARS:

4.0

PROFESSIONAL:

4.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The broad objective of this project is to gain further understanding regarding the endocrinology of hormonal responses in women in order to provide rational design of therapies directed toward the clinical problems of endometriosis, pregnancy loss, fertility, and estrogen-dependent reproductive neoplasms. Advances during the current period include: 1) isolation of a novel RXR-binding protein from a breast cancer cDNA expression library; 2) demonstration that the novel RXR binding protein is present in breast cancer tissues and binds to the retinoid X receptor; 3) determination that mRNA for the novel protein is expressed in breast cancer cells in addition to other reproductive tissues; 4) demonstration that the novel protein and RXR interact *in vivo* using the dual hybrid yeast system; 5) demonstration that the retinoid X receptor interacts with the general transcription factor TFIIB in a yeast system; 6) determination of the regions required for RXR binding to TFIIB using GST binding assays and co-immunoprecipitation studies; 7) a demonstration that the interaction between RXR and the general transcription factor IIB requires ligand, and 8) demonstration of RXR activation of estrogen responsive genes in the presence of the peroxisome proliferator-activated receptor (PPAR). We plan to further characterize the novel RXR-binding protein and study additional proteins that may contribute to modulation of estrogen responsive genes.

PROJECT DESCRIPTION:

OBJECTIVE: The overall objective remains unchanged during the current period. Our objective continues to be to gain a further understanding of the role of the retinoid X receptor in the modulation of estrogen responsive genes. This objective is approached in order to understand the mechanisms involved in tissue specific regulation of nuclear hormone receptor function. As proposed, research during the current period focused on the study of proteins other than the estrogen receptor which may contribute to regulation of estrogen responsive tissues. In addition we tested naturally occurring estrogen responsive promoters by regulation of the retinoid X receptor and PPAR. Lastly, use of the dual hybrid yeast system has begun to permit characterization, isolation, and functional study of RXR action and modulation of the receptor action by RXR-binding proteins. During the current period we have: 1) continued to characterize the role of the PPAR modulation of estrogen responsive genes; 2) isolated a novel RXR binding protein from a breast cancer cDNA library; 3) demonstrated that the retinoid X receptor functions in part by binding to the general transcription factor TFIIB.

METHODS:

Cotransfection assays were used to test for modulation of estrogen responsive genes by nuclear hormone receptors. Binding of receptors was tested using baculovirus and bacterial expressed proteins in addition to *in vitro* translated receptors. Further, a DNA receptor precipitation assay was developed which permitted a demonstration of RXR receptor mutant binding to estrogen responsive elements.

Further, using a method of non-radioactive detection of receptor protein complexes from lambda phage expression library, a protein binding to the retinoid X receptor was identified. Far Western analysis was also used to test binding of this protein to the retinoid X receptor and to proteins present in nuclear extracts prepared from breast cancer cells.

In addition to the above assays, levels of receptor were studied with standard Western, Northern, Southern, and mobility shift analysis.

MAJOR FINDINGS:

During the period we found that nuclear extracts prepared from breast cancer cells express several RXR binding proteins with varied molecular weights of approximately a 160, 140, 120, 70 and 30 KD molecular weight. Using an expression library prepared from breast cancer mRNA and the non-radioactive detection method for receptor protein interaction described last period, we isolated a cDNA clone expressing a protein which bound to the retinoid X receptor. Analysis from the published sequences present in the gene sequence data banks show the gene to be novel. Further, binding studies using both *in vitro* translated proteins as well as baculovirus expressed proteins confirmed the binding of the protein to the RXR receptor expressed in recombinant form. In addition Northern analysis confirmed expression of the mRNA in breast cancer tissues as well as other reproductive tissues. Mapping studies confirmed specific regions of the novel protein were required for binding to the retinoid X receptor. Using serial screening of library and overlapping clones, the entire cDNA encoding the mRNA was isolated.

Further, the retinoid X receptor was expressed in yeast using the dual hybrid yeast system. Analysis confirmed activation of the receptor which was ligand dependent. Further analysis showed evidence of binding to the newly characterized novel RXR binding protein. This suggests that the interaction between the newly identified protein species and RXR can occur *in vivo*.

In a related project, the function of RXR was studied in detail using the yeast dual hybrid system. RXR was noted to interact with the general transcription factor, TFIIB, in a ligand dependent fashion. Cotransfection assays performed in P-19 embryonal carcinoma cell lines confirmed activation in undifferentiated cells as well. Using GST binding studies and coprecipitation studies, the regions required for interaction between the basal



transcription factor, TFIIB and the retinoid X receptor were identified and characterized. It was determined that the carboxyl region of RXR is required for binding to the transcription factor TFIIB. Furthermore, a detailed analysis using both functional and binding studies revealed that, while binding was necessary for TFIIB-dependent activation, binding alone was not sufficient for RXR to increase reporter activity. This observation suggests an additional requirement for activation by the transcription factor TFIIB in addition to receptor binding.

In addition to the above findings, studies have been performed with the peroxisome proliferator activated receptor. These studies have shown that the PPAR RXR heterodimeric pair noted (in prior reports) is able to bind a naturally occurring estrogen response element, specifically of that present in the oxytocin gene. Furthermore, cotransfection studies have demonstrated that addition of RXR and PPAR confer a ligand dependent responsiveness to this naturally occurring promoter.

SIGNIFICANCE TO BIOMEDICAL RESEARCH IN THE PROGRAM OF THE INSTITUTE:

A characteristic of reproductive tissues is their ability to respond to steroid hormones, however, it is not known how signals for these hormones are modulated at the tissue level. That is, addition of the anti-estrogen tamoxifen works as a anti-estrogen at the level of the breast, however, it is stimulatory to the endometrium. This paradox has puzzled reproductive scientists. Since we have previously shown that the retinoid X receptor was capable of modulating estrogen responsive genes, we have tested the hypothesis that the retinoid X receptor may be modulated at the tissue specific level by specific factors expressed in reproductive tissues. To identify such factors we have used a protein interaction cloning strategy to isolate a novel RXR binding protein present in breast cancer tissues. Breast cancer is a major public health concern that will affect 1 in 9 women. In addition to relevance to breast cancer, the present work contributes to an understanding of the modulation of nuclear hormone receptor function at the level of reproductive tissues. Thus the work may have relevance beyond a specific tumor and may be of more generalized significance.

Further, an understanding of the function of the retinoid X receptor is inherently interesting since little is known about the relevance of gene regulation by this receptor to reproductive function.

The demonstration that the retinoid X receptor is capable of binding to the transcription factor TFIIB furthers knowledge regarding receptor-mediated gene activation. In addition to previously published methods of testing for receptor interaction with basal transcription factors, we have used the dual hybrid yeast system which permits testing of the significance of this interaction *in vivo*. The *in vivo* demonstration of a receptor interaction with a general transcription factor in the yeast cells is unique. Our findings suggest that the retinoid X receptor is capable of interacting with other transcription factors present in yeast which can compete for interaction with TFIIB. Further, the demonstration of the interaction of the retinoid X receptor with TFIIB suggests that (since RXR contributes to heterodimeric interactions of receptors on particular hormone response elements) in some cases both members of a heterodimeric receptor pair are able to interact with the basal transcription complex via TFIIB. Thus RXR may not simply function to increase or augment DNA binding, but rather contribute to activation of basal transcription machinery. In addition, expression of the retinoid X receptor in yeast permits functional analysis in a system devoid of endogenous retinoid X receptor enabling our group to clearly define the regions of the retinoid X receptor involved in interaction with other hormone receptors as well as with other interacting proteins and basal transcription factors. Additional studies are required to further define receptor interactions using this system.

PROPOSED COURSE:

We plan to continue to study and characterize the novel RXR binding protein which is expressed in reproductive tissues. Specifically, we will determine the nature of this protein, the possible relevance of this protein to oncogenesis in reproductive tissues, and further characterize the regions and domains of this novel protein with regard to function. In addition, we plan to test naturally occurring estrogen responsive and retinoid X responsive genes for regulation by this novel receptor binding protein. Further, we plan to study endometrium and reproductive



tissues for expression of this novel retinoid X receptor binding protein in an effort to link expression with function.

Our second aim is to continue to study the specific function of the retinoid X receptors in reproductive tissues. To accomplish this goal we will continue to utilize the dual hybrid yeast system to characterize the function of the retinoid X receptor in a system devoid of endogenous RXR expression. Additional efforts will be made to understand the factors that modulate expression of the retinoid X receptor in reproductive tissues.

PUBLICATIONS:

Chudacoff R, Alexander J, Alvero R, Segars JH. A modified method of tissue expansion vaginoplasty for treatment of congenital vaginal agenesis, *Obstetrics and Gynecology*, in press.

Leong G, Blanco JG, Rolfes R, Marton M, Wang K, Wang IM, Moehle C, Tan A, Ozato K, Segars JH. Examination of the interaction between the retinoid X receptor and the general transcription factor IIB (TFIIB) in the dual hybrid yeast system, *Endocrinology* under 35, in press.

Nunez SB, Medin JA, Keller H, Wang K, Ozato K, Whali W, Segars JH. Retinoid X receptor β and the peroxisome proliferator-activated receptor activate an estrogen response element, *Rec Prog Horm Res* 1995;50:409.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00637-02 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Steroid Hormone Action in Female Reproduction

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.	L.K. Nieman	Research Medical Officer	URM, SGR, DEB, NICHD
Others:	T. Rarick	Medical Staff Fellow	LTPB, NICHD
	J. Segars	Sr. Clinical Investigator	MMTP, SGR, DEB, NICHD
	T. Kim	Special Volunteer	URM, SGR, DEB, NICHD
	P. Driggers	Senior Staff Fellow	MMTP, DEB, NICHD
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COOPERATING UNITS (if any)

Maria Merino, M.D., Clinical Pathology, NICHD; Nancy Alexander, Ph.D., Contraceptive Development Branch, NICHD

LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Gynecologic Research, Unit on Reproductive Medicine

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this project is to understand better the development of the endometrium and oocyte in women and to investigate the role of gonadal steroids in these processes. Studies evaluating ovarian function in women suggested that growth hormone (GH) may be an important mediator of follicular development, as its receptor is present in the granulosa cells of the developing follicle and in the corpus luteum, the estrogen and progesterone steroidogenic compartments. We also demonstrated that a complete fast for 72 hours during the mid-follicular phase does not disrupt folliculogenesis, ovulation, or menstrual cycle dynamics in normal weight women, suggesting that brief periods of undernutrition are not deleterious to reproductive function. Another study evaluated the effect of aging on reproductive function. FSH was increased, and inhibin decreased, in the luteal-follicular transition of women aged 40 - 50 years compared to those aged 20 - 30 years, but the incidence of abnormal endometrial biopsies was similar. These results suggest that follicular recruitment, but not luteal function or endometrial maturation, is disturbed in cycling women over 40 years old and may contribute to decreased fertility.

The process of uterine remodeling that is so remarkable in the mammalian uterus is achieved through coordinated proliferation, differentiation and cell death. While the gonadal steroids estradiol and progesterone appear to be required for these organ-specific processes, the mechanism(s) by which these processes are regulated remain unclear. Potential mediators of estrogen action include IGF-I and c-Myc, a transcription factor known to be induced by growth factors. We used a well-characterized in vivo ovariectomized mouse model to correlate sex steroid-induced proliferation with the induction of IGF-1 and c-Myc mRNA in the uterine endometrium. We have found that IGF-I and c-Myc levels parallel each other and the estrogen-induced processes of differentiation in this model, suggesting that they are important to proliferation. The induction of IGF-I by GH in other tissues prompted an evaluation of whether the GH receptor was present in the endometrium, based on the hypothesis that GH might act directly on the endometrium and so modulate estrogen effect. GH receptor was found to be present throughout the endometrium, but was not regulated by estradiol.

Project Description:Objectives:

Our long-term goal is to investigate infertility, to develop new contraceptives, and to evaluate new approaches to therapy of the menopause. Recent studies have sought to understand better the process of folliculogenesis and the role of growth hormone and IGF-1 in endometrial growth and development.

We have investigated the effects of nutrition and aging on the process of folliculogenesis. In men and monkeys, caloric deprivation adversely affects reproductive function. As many women severely limit caloric intake for weight reduction, a similar effect of fasting on folliculogenesis might have important implications for women desiring fertility. Also, many women desire fertility after the age of 40, yet fecundity decreases at that time. We investigated hormonal dynamics and endometrial morphology in older women to understand better this decline in reproductive function.

The process of uterine remodeling that is so remarkable in the mammalian uterus is achieved through coordinated proliferation, differentiation and cell death. While the gonadal steroids estradiol and progesterone are required for these organ-specific processes, the mechanism(s) by which these processes are regulated remain unclear. We postulate that IGF-1 and c-myc mediate estrogen's mitogenic effects and that growth hormone (GH) as well as estrogen might induce endometrial IGF-1 and mitogenesis. Observations that growth hormone increases uterine weight independent of estradiol in hypophysectomized rats and is associated with an increased incidence of fibroids in acromegalic women support this idea. We thus examined whether the GH receptor is present in estrogen treated murine uterus, and in fibroid tumors from women.

Methods employed:

Effects of age on luteal function and endometrial maturation: Thirty-two regularly cycling women aged 20 to 30 or 40 to 50 years, had daily blood drawing starting on cycle day 6 to 10 and continuing until 2 days after the onset of next menses for LH, FSH, estradiol, inhibin, PP14 and progesterone measurements. In addition, 60 women, aged 20 to 30 or 40 to 50 years, had a total of 93 endometrial biopsies performed on day 7 to 9 after the LH surge for histologic dating.

Evaluation of the effects of fasting on folliculogenesis in women: Twelve women were randomly fed or fasted on cycle days 7 to 9 to investigate whether fasting for 72 hours might disrupt reproductive function in normally cycling women, as it does in men and monkeys. Gonadotropin secretion, follicle development by ultrasound, and follicular and luteal phase lengths were measured.

GH receptor in normal ovaries and leiomyoma: Ovaries were obtained at the time of surgery from non-pregnant normally cycling women undergoing hysterectomy/oophorectomy for non-ovarian gynecological indications. Leiomyomata and surrounding myometria were obtained from women who did or did not receive preoperative GnRHa therapy. Tissues were processed for mRNA extraction and PCR detection of the GH receptor and GAPDH, and for in situ hybridization.

Animal studies: An oophorectomized murine model was used to examine whether IGF-1 and c-myc might mediate gonadal steroid action in the uterine glands and stroma. In situ hybridization for IGF-1 and c-myc mRNA was correlated with mitotic index after direct Alzet pump infusion of IGF-1 into the cornua, or after subcutaneous administration of RU 486, progesterone and/or



estrogen. A second study evaluated the presence of growth hormone receptor in this model using PCR and in situ hybridization.

Major Findings:

1. Effects of aging on folliculogenesis and endometrial development: Serum FSH levels were increased whereas inhibin concentrations were reduced in the luteal-follicular transition of women > 40 years. No other hormonal changes were seen in this population, including progesterone and PP14 secretion. Disruption of endometrial maturation occurred at a similar frequency in both age groups. These results suggest that follicular recruitment, but not luteal function or endometrial maturation, is disturbed in cycling women over 40 years old and may contribute to the decline in fertility with aging.

2. Evaluation of the effects of fasting on folliculogenesis in women: None of the outcome measures differed between the fed and fasted cycles. We conclude that the reproductive axis of normally cycling women of mature gynecologic age and > 20 % body fat is not adversely affected by a 3 day fast during the mid-follicular phase. These data contrast with the reported sensitivity of the hypothalamic-pituitary-gonadal axis to acute nutritional withdrawal in men and monkeys and may reflect different sensing of fuel availability in women, possibly mediated through a higher percent body fat.

3. Ovarian production of GH receptor: PCR and in situ hybridization confirmed the presence of GH receptor mRNA in corpora lutea and granulosa cells of antral follicles, but not in stroma, theca cells or primordial follicles of 7 surgically-removed ovaries from normally cycling women. Interestingly, not all follicles stained for GH receptor, suggesting that its expression may be associated with specific physiologic states, such as active steroidogenesis, and be absent in others, such as atresia.

4. Presence of GH receptor in leiomyoma: GH receptor mRNA was present in both leiomyoma and surrounding myometrium with greater expression in leiomyoma, suggesting that GH may act directly on the human uterus. GnRHa treatment reduced GH receptor gene expression, implying that GH receptor expression in leiomyoma may be regulated by sex steroids.

5. Animal studies: After gonadal steroid treatment, endometrial IGF-1 and c-myc mRNA expression increased, and paralleled the pattern of differentiation and proliferation. Treatment with estrogen (alone and with progesterone and RU 486) induced proliferation of the epithelial cells, while the addition of progesterone shifted mitosis to the stroma and resulted in differentiation of the epithelium. IGF-1 and c-myc showed coordinate expression in the epithelium (estrogen treatment) or both compartments (estrogen and progesterone treatment). The c-myc expression after IGF-1 alone was similar to that seen with estrogen. These preliminary findings suggest a role for IGF-1 as an estromedin.

PCR and in situ hybridization demonstrated GH receptor in the murine endometrium. There was no specific regulation by estradiol, suggesting that GH receptor expression is constitutive and is not involved in modulation of gonadal steroid action.

Significance to Biomedical Research and the Program of the Institute:

These studies reflect the NICHD mission to understand better the physiology of normal and abnormal reproductive states and to develop contraceptive agents. The ability of the uterus to undergo coordinated timely growth and regression provides an excellent model to understand the influence of growth factors and protooncogenes on cell cycle progression and the way in which hyperplasia and uncontrolled cell growth is prevented in vivo.



Proposed Course:

1. Characterization of progesterone-dependent endometrial products. We hypothesize that progesterone-induced peptides are required for endometrial epithelial differentiation and hence, implantation. We will evaluate progesterone regulation of three candidate peptides: c-MET, the vitronectin receptor, and smooth muscle myosin II. Activation of the MET receptor by mesenchymally derived hepatic growth factor is an integral element of epithelial cell differentiation in other systems, but has not been examined in the uterus. The morphologic association of stroma and epithelium, and the coordinated growth of the endometrium after menses, suggest that hepatic growth factor may play a role in this process. The vitronectin receptor, comprised of the pair, $\alpha v \beta 3$, is an integrin that binds ligands on the trophoblast surface, and can be blocked with peptides containing the tripeptide RGD (Arg-Gly-Asp), which prevent implantation in the mouse. This integrin is present on epithelial cells on days 19 to 24 and is absent in up to 20% of infertile women with delayed mid-luteal endometrial development and endometriosis-associated infertility. Its regulation by progesterone is not known. Smooth muscle myosin II (SMMII) is expressed in glandular epithelial cells in the midluteal phase in the intact baboon, and is induced by estrogen and progesterone treatment in ovariectomized animals. We are unaware of studies of this peptide in human endometrium.

Two experimental models will be used: in vitro culture of Ishikawa cells, an hormonally-responsive endometrial epithelial cell line, and examination of endometrial biopsies obtained from normally cycling women.

Ishikawa cells will be grown in standard media containing fetal calf serum. To examine the effects of progesterone, estrogen, and antiproggestins on the expression of the candidate peptides, cells will be grown in media containing physiologic amounts of these compounds (estradiol 150 pg/mL; progesterone 20 pg/mL; RU 486 20 pg/mL) for 48 hours, and then harvested and processed for in situ hybridization or immunohistochemistry. To evaluate progesterone induction of other peptides, we will use mRNA from these treatment groups and the technique of differential display to identify potential progesterone-dependent epithelial products without ascertainment bias.

Normally cycling women will be recruited to undergo an endometrial biopsy, blood sampling and ovarian ultrasound. All cycles will be characterized hormonally by determination of the LH surge and by measurement of estradiol and progesterone. Follicular phase biopsies will be correlated with the size of the dominant follicle by ultrasound. Luteal phase biopsies will be timed according to the LH surge and will be obtained using a Pipelle curette. A part of each specimen will be placed in formalin for dating by Noyes' criteria. The remaining tissue will be used for study procedures, including in situ hybridization or immunohistochemistry to localize the candidate peptides to epithelial and/or stromal cells and to correlate changing patterns of expression over the menstrual cycle with hormone levels. The DNA sequence of the candidate peptides is known, and standards and antibodies to each are in use in our laboratory or available to us.

2. The potential contraceptive action of low dose antiproggestins: We previously showed that RU 486 can retard endometrial maturation in women and inhibit implantation in the guinea pig, suggesting that antiproggestins may be effective contraceptive agents that render the endometrium hostile to implantation without affecting hormonal rhythm. Further development of these properties of antiproggestins would represent a significant new advance in contraceptive technology.

RU 486 is no longer available from Roussel-UCLAF. However, the Contraceptive Development Branch, NICHD, has another antiproggestin, CDB 2914. Results from pre-clinical studies suggest that its activity is similar to that of RU 486. We will develop this compound for extramural and intramural clinical trials, beginning with protocol 95-CH-0168, "Dose-response Relationships for the Antiproggestin CDB 2914 in

Cycling Women". Women will receive the compound in the luteal phase in this phase 1-2 trial, to determine safety and to provide initial information about biologic effects. Blood will be obtained for pharmacokinetic studies, and for evaluation of changes in cortisol, ACTH, LH, FSH, estradiol and progesterone. Women will chart menses and basal body temperature to evaluate antagonism of endometrial or hypothalamic progesterone action. Should the compound appear promising, another protocol will be proposed to include endometrial biopsy, which will provide placebo or antiprogesterin-exposed tissue to test whether endometrial candidate peptides are regulated by progesterone.

Publications:

Batista MC, Cartledge TP, Zellmer AW, Merino MJ, Axiotis C, Bremner WJ, Nieman L. Effects of aging on menstrual cycle hormones and endometrial maturation, *Fertil Steril* 1995;64:492-299.

Nieman LK. Estrogens and progestins. In: C Smith & A Reynard, eds. *Essentials of Pharmacology*. Philadelphia:WB Saunders, 1995;563-573.

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Sharara FI & Nieman LK. Growth hormone receptor mRNA expression in leiomyoma and surrounding myometrium, *Am J Obstet Gynecol*, in press.

Sharara FI & Nieman LK. Identification and cellular localization of growth hormone receptor gene expression in the human ovary, *J Clin Endocrinol Metab* 1994;79:670-672.

Sharara FI, Bhartiya D, Nieman LK. Growth hormone receptor gene expression in the mouse uterus: modulation by gonadal steroids, *J Soc Gynecol Invest* 1995;1:285-9.

Patents:

none

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD-00638-02 DEB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Physiology of Hypercortisolism

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

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LAB/BRANCH

Developmental Endocrinology Branch

SECTION

Section on Gynecologic Research, Unit on Reproductive Medicine

INSTITUTE AND LOCATION

National Institute of Child Health and Human Development

TOTAL STAFF YEARS:

1.5

PROFESSIONAL:

1.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Cushing syndrome, a fatal disease, is suspected in many thousands of patients each year, but confirmed in only a fraction of these. This project seeks to identify accurately which patients have Cushing syndrome, to define the etiology of their disease and to treat it optimally.

A major initiative in the past year has been to improve the approach to the differential diagnosis of Cushing syndrome. This effort included development of optimal criteria for interpretation of diagnostic tests that maintain 100% specificity for the diagnosis of Cushing's disease. We compared the performance of the 8 mg overnight dexamethasone suppression test in 41 patients who also received the traditional 6 day test and identified the best timepoints at which to measure cortisol for optimal diagnostic accuracy. We achieved 65 - 70% sensitivity using refined criteria for the traditional and overnight tests, and a 91% sensitivity if the tests were combined. Similar approach was used to optimize the traditional 4 day metyrapone stimulation test to develop criteria for 100% specificity in 185 patients with ACTH-dependent Cushing's syndrome. This test yielded a sensitivity of 72% using urine 17-hydroxysteroid and plasma 11-deoxycortisol endpoints. When combined with the traditional dexamethasone suppression test, sensitivity increased to 88%. These studies indicated that tests for the differential diagnosis of Cushing's syndrome can be combined to give improved sensitivity at 100% specificity. Further work will define an optimal cost-effective diagnostic strategy. In response to a report that venous sampling from the cavernous sinuses is superior to CRH-stimulated inferior petrosal sinus sampling, we also evaluated this test in 15 patients. In our hands, cavernous sinus sampling failed to diagnose correctly 20% of patients, while inferior petrosal sinus sampling correctly identified Cushing's disease in all patients.

Project Description

Objectives:

This project seeks to identify accurately which patients have Cushing's syndrome, to define the etiology of their disease, and to treat it optimally. Cushing's syndrome, a fatal disease, is suspected in many thousands of patients each year, but confirmed in only a fraction of these. Once identified, its cause must be found and successfully treated. The identification, differential diagnosis and treatment of this syndrome pose problems for the clinician.

There is no test that reliably separates individuals with pseudo-Cushing's states, who have mild hypercortisolism and minimal physical features of Cushing's syndrome, from those with mild or intermittent Cushing's syndrome, who may present in an identical fashion. We are exploiting the different pathophysiology in the pseudo-Cushing's and Cushing's syndrome states to develop new tests for this distinction. Patients with Cushing's syndrome have either an ACTH-dependent abnormality (corticotrope adenoma or ectopic ACTH-secreting tumor), or a primary adrenal tumor; in this setting the CRH neuron is appropriately suppressed. In contrast, patients with pseudo-Cushing's syndrome have an hypothalamic abnormality, hypersecretion of CRH; the pituitary and adrenal glands are normal. As a result of these differences, we hypothesize that patients with pseudo-Cushing's syndrome might maintain a normal diurnal pattern of cortisol secretion, in distinction to the invariant cortisol levels seen in patients with Cushing's syndrome.

Our second initiative is to improve the approach to the differential diagnosis and treatment of the causes of Cushing's syndrome. The differential diagnosis of Cushing's syndrome, especially the ACTH-dependent forms, is difficult. The treatment of ectopic ACTH secretion depends on localization and surgical removal of the tumor, which may be occult in up to 50% of cases.

Methods employed:

The methods used in these clinical investigations involve blood and urine collection for hormone measurements by RIA with and without administration of a variety of provocative agents. Standard radiographic studies and catheterization procedures are performed to identify a tumor. The diagnosis of pseudo-Cushing's syndrome is confirmed by long-term physical and biochemical evaluation that fails to demonstrate convincing evidence for evolving Cushing's syndrome. The diagnosis of Cushing's syndrome is confirmed by physical examination and standard biochemical criteria; an etiologic diagnosis is assigned only after surgical cure or histopathologic identification of an ACTH-containing or primary adrenal tumor.

Major Findings:

Differential Diagnosis of Cushing's syndrome. We continue to evaluate tests for the differential diagnosis of Cushing's syndrome. We compared the performance of the standard 6 day, 8 mg, dexamethasone suppression test with the 4 day metyrapone stimulation test and the 8 mg overnight dexamethasone suppression test. Our eventual aim is to develop a decision tree based on the individual and combined diagnostic accuracy of these and other tests (CRH stimulation and inferior petrosal sinus sampling). In response to a report that venous sampling from the cavernous sinuses is superior to CRH-stimulated inferior petrosal sinus sampling (J Clin Endocrinol Metab 76:637-641, 1993), we also evaluated this test.

1. Overnight dexamethasone suppression test: We compared the performance of the 8 mg overnight dexamethasone suppression test in patients who also received the traditional 6 day test and identified the best timepoints at which to measure cortisol for optimal diagnostic accuracy.

Various pre- and post-dexamethasone time points were examined and the test was optimized as follows: blood

is drawn for cortisol determination at 0900h before and 0830h after administration of dexamethasone, 8 mg, at 2300h. Using these time points, we found that a > 69% suppression of plasma cortisol yielded a 71% sensitivity and 100% specificity in 41 patients (34 Cushing's disease; 7 ectopic ACTH secretion). When compared to the criterion developed previously by Tyrrell et al. (Ann Intern Med 104:180, 1986; suppression of plasma cortisol > 50% at time combination 0800h before and after dexamethasone), the sensitivity was 88%, but the specificity decreased to 57%. To achieve 100% specificity of the test with the 0800h sampling times, a > 80% suppression of plasma cortisol was required in our patients, which dropped the test sensitivity to 59%. This study demonstrates the importance of evaluating a large number of patients so that comparison groups of appropriate size are available. A similar sensitivity and specificity (100%) were achieved with the standard 6 day test, using criteria of > 69% suppression of 17-hydroxysteroid excretion (sensitivity 68%) or > 90% suppression of urine cortisol excretion (sensitivity, 65%).

2. Metyrapone stimulation test: We compared the traditional 4 day metyrapone stimulation test to develop criteria for 100% specificity in 185 patients with ACTH-dependent Cushing's syndrome. We found that the test could be reduced to two days: the day before and during metyrapone administration (750 mg every 4 hours, for six doses, beginning at 0800h on the second day). Urine is collected on both days, beginning at 0800h, for 17-hydroxysteroid measurement. Plasma 11-deoxycortisol is measured at 0800 at 0 and 24 hours (before metyrapone), and at 48 hours (after metyrapone), and the difference between the post- and the mean pre-metyrapone values is calculated. Optimal criteria for the diagnosis of Cushing's disease were a 400-fold increase in 11-deoxycortisol and a > 70% stimulation in 17-hydroxysteroid excretion. When used together, a positive response to either criterion yielded 72% sensitivity at 100% specificity. When combined with the standard dexamethasone suppression test, using the criteria above, the sensitivity increased to 88%.

3. Cavernous sinus sampling vs. inferior petrosal sinus sampling: During a single sampling procedure, ACTH levels were compared in simultaneous samples obtained using tracker catheters inserted into the cavernous sinuses, and in simultaneous samples obtained from both petrosal sinuses before and after administration of CRH. 15 patients were studied prior to surgical cure of Cushing's disease. We found that unstimulated levels of ACTH in the cavernous sinuses were higher than unstimulated levels of ACTH in the petrosal sinuses, but 3 of 15 patients failed to show sufficient central-to-peripheral cavernous sinus ratios and thus were falsely negative for the diagnosis of Cushing's disease (test sensitivity, 80%). By comparison, the test sensitivity for the petrosal sinus samples was 87% for the unstimulated samples and 100% for the CRH-stimulated samples. Because of the 20% false negative rate, we do not recommend that cavernous sinus sampling without CRH be used for the differential diagnosis of ACTH-dependent hypercortisolism.

4. Pediatric Cushing's syndrome: We analyzed the clinical presentation, diagnostic evaluation, and treatment of 59 patients with Cushing's syndrome between the ages of 4 and 20 years, admitted to the NIH. Fifty had Cushing's disease, six had primary adrenal disease, and three had ectopic ACTH secretion. Of those with Cushing's disease, magnetic resonance imaging of the pituitary indicated a tumor in only 52%. Transsphenoidal surgery was curative in 48 of 49 who underwent transsphenoidal exploration. We showed that previously developed criteria for interpretation of the CRH test and inferior petrosal sinus sampling were useful in children.

Significance to Biomedical Research and the Program of the Institute:

Development of improved tests for the diagnosis of Cushing's syndrome has a great potential impact on public health. In our experience, the greatest challenge in the evaluation of patients with possible Cushing's syndrome is to establish the diagnosis. Cushing's syndrome is considered, but is statistically improbable, in thousands of patients each year. The currently available tests do not minimize equivocal information and may lead to a mistaken diagnosis of Cushing's syndrome, which prompts further tests and places the patient at risk for inappropriate intervention, while increasing health care expenditure. Our previous finding that the classic

screening test, UFC excretion, has a significant false negative and false positive rate illustrates the importance of continued efforts to improve both the sensitivity and the specificity of screening tests.

The above effort to better discriminate pseudo-Cushing's syndrome may impact on tens of thousands of patients each year. In contrast, our effort to refine the approach to the differential diagnosis of Cushing's syndrome has a smaller overall benefit to the public health. However, we are now recognized as one of a handful of centers in the world with experience of more than 200 cases, and so we have a unique opportunity to develop an accurate, cost-effective diagnostic strategy that can be used by others with less experience. The benefits to the public health of this large ongoing experience are illustrated in the studies cited in this report. Many of the tests for the differential diagnosis of Cushing's syndrome have been developed in small groups of patients, with very few of the important comparison groups, such as those with ectopic ACTH secretion. Our ongoing analysis of a large group of patients has enabled us to simplify, standardize and set criteria for the interpretation of these tests. Also, by quickly evaluating reports advocating new tests based on small numbers of patients, we help to establish whether the new tests have value in a larger population. Similarly, by virtue of its unique size, our report of a large number of pediatric patients with Cushing's syndrome revealed that ectopic ACTH secretion must be considered in this population, and also showed the unrecognized utility of transsphenoidal surgery in these patients.

Proposed Course:

Diagnosis of Cushing's syndrome. We will continue to measure diurnal plasma cortisol to assess its power to discriminate between pseudo-Cushing's syndrome and Cushing's syndrome. Although preliminary data indicate that this test may have a high diagnostic accuracy, it is not practical, so we plan to obtain salivary cortisol measurements. We anticipate this change in methodology will improve the test's sensitivity, as hypercortisolism suppresses CBG and increases free cortisol, the fraction measured in saliva. It also will make the test convenient and feasible for in-home use, further decreasing the cost of evaluation for Cushing's syndrome. We plan to extend our previous observation that dexamethasone pre-treatment suppresses the response to an CRH stimulation test by administering dexamethasone or placebo prior to inferior petrosal sinus sampling in normal individuals and those with pseudo-Cushing's or Cushing's syndrome. This will allow us to establish the range of basal and CRH-stimulated ACTH levels (with and without dexamethasone) in these groups; minimal overlap may suggest this as a diagnostic approach, especially for patients with mild hypercortisolism. Even when it is clear that these patients have Cushing's syndrome, the interpretation of differential diagnostic tests is suspect because of the concern that the corticotropes are inadequately suppressed.

Differential Diagnosis of Cushing's syndrome. The availability of CRH and the development of IPSS and CRH testing has revolutionized the differential diagnosis of Cushing's syndrome, leading to near 100% sensitivity and specificity in our hands. It is unlikely, however, that IPSS will become widely available, since significant catheterization experience is necessary for its success. Additionally, it has a small, but measurable morbidity, and there is no consensus as to its appropriate role in the diagnostic strategy for Cushing's syndrome. We continue, therefore, to analyze the performance of all tests, with the goal of developing a simple and convenient decision tree for the evaluation of Cushing's syndrome that minimizes cost without compromising diagnostic accuracy.

Because inferior petrosal sinus sampling is dependent on the skill of the radiologist, it is not widely available. Additionally, the morbidity of cerebrovascular accident attendant to the procedure is probably related to occlusion of small vessels. For these reasons, we are interested in exploring the diagnostic utility of jugular venous sampling for measurement of CRH-stimulated ACTH levels. We hypothesize that this procedure may have a diagnostic accuracy similar to that of inferior petrosal sinus sampling. It would have the advantage of being easier to perform, thus more widely available, and might also be more safe. We plan to compare the two procedures in 30 patients.

Unfortunately, the localization of ectopic ACTH-secreting tumors fails initially in 50% of these patients, and may not be achieved for 20 years, exposing them to the deleterious effects of hypercortisolism and the risk of metastatic disease. Thus, we plan to focus on promising venues for the localization of these tumors. We will evaluate the utility of radionuclide-labeled sandostatin, a somatostatin analog, for the detection of ectopic ACTH-secreting tumors, and will explore the feasibility of total body PET scan for identification of intrathoracic lesions.

The rate of recurrence in patients successfully "cured" of Cushing's disease by transsphenoidal surgery has not been established, but may be as great as 10%. We continue to follow our cohort to determine the risk of recurrence in the group and are extending our earlier observation that the response to CRH in the post-operative period may predict those patients destined to recur. Should this be clearly established in a large group of patients, strong consideration may be given to early x-irradiation therapy in patients with abnormal post-operative CRH responses.

Publications:

Avgerinos PC, Yanovski JA, Oldfield EH, Nieman LK, Cutler GB Jr. The metyrapone and dexamethasone suppression tests for the differential diagnosis of Cushing's syndrome: a comparison, *Ann Int Med* 1994;121:318-27.

Dichek HL, Nieman LK, Oldfield EH, Pass HI, Malley JD, Cutler GB Jr. A comparison of the standard high-dose dexamethasone suppression test and the overnight 8-mg dexamethasone suppression test for the differential diagnosis of Cushing's syndrome, *J Clin Endocrinol Metab* 1994;78:418-422.

Doppman JL, Nieman LK, Chang R, Yanovski J, Cutler GB Jr, Chrousos GP, Oldfield EH. Selective venous sampling from the cavernous sinuses is not a more reliable technique than sampling from the inferior petrosal sinuses in Cushing's syndrome, *J Clin Endocrinol Metab* 1995;80:2485-2489.

Magiakou MA, Mastorakos G, Gomez MT, Doppman JL, Cutler GB Jr, Oldfield EH, Nieman LK, Chrousos GP. The NIH experience with Cushing's syndrome in children and adolescents: Presentation, diagnosis and therapy, *N Engl J Med* 1994;331:629-636.

Nieman LK, Cutler GB Jr. Cushing's Syndrome. In: DeGroot L, ed. *Textbook of Endocrinology*. Philadelphia:WB Saunders, 1994;1741-1769.

Patents:

none

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00841-13 BB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Methods for Comparing and Analyzing Data from Several Complex Surveys

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
Others:	H.J. Hoffman	Chief, Epidemiology	OD, EB, NIDCD
	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD
	Y.J. Lee	Chief	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

BB, CTS, DCPC, NCI (B. Graubard); BRB, NCI (E. Korn)

LAB/BRANCH

Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.0

PROFESSIONAL:

0.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human ☐ (b) Human ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00855-04 BB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Methods for the Common Odds Ratio of a Number of Contingency Tables

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: K.F. Yu Mathematical Statistician BMSB, DESPR, NICHD

Others: A.A. Herman Visiting Scientist EB, DESPR, NICHD
J.F. Troendle Senior Staff Fellow BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.35

PROFESSIONAL:

0.35

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human ☐ (b) Human ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study will develop statistical methods for analyzing categorical data, particularly in the comparison of different groups. Examples are the common odds ratios for a number of contingency tables and the standardized risk difference in the comparison of two groups stratified by a covariate. The optimality properties of the developed methodology will be investigated and established. The likelihood equations are to be derived. The newly developed, maximum likelihood and conditional maximum likelihood methodologies are to be examined. The study has been extended to do a comparison of a number of contingency tables in the form of estimation and hypothesis testing. This methodology is particularly useful in detecting the adequacy of matching in the case-control epidemiological studies. All these methodologies have wide applications to epidemiological studies, biomedical studies, genetics and other disciplines. Examples include testing the Hardy-Weinberg Law across strata in genetics and comparing the black-white differences in birth specific infant mortality curves.

Project Description:

Objectives: To develop statistical methods for estimating the odds ratio of a number of contingency tables and a family of standardized risk difference parameters. To develop statistical methods for testing hypotheses concerning a number of contingency tables. To study black-white differences in birth weight specific mortality curves. To study the Hardy-Weinberg Law across strata.

Methods Employed: Classical and modern statistical and probabilistic methods are employed to analyze the problems. New methodology is also developed to handle the problems. Computer and numerical techniques are utilized.

Major Findings: An estimator proposed by Greenland and Holland (1991, Biometrics 47, 319-322) for a standardized risk difference parameter is shown to be a maximum likelihood estimator if the consistent estimator of the common odds ratio is appropriately chosen. The statistical problem under consideration is reparameterized for a better understanding. Likelihood equations are derived. A family of easily computable estimators of the common odds ratio of a number of contingency tables is derived from a set of reasonable postulates. A necessary and sufficient condition has been discovered for the strong consistency of this family of estimators. Also, a strongly consistent estimator has been found for the asymptotic variance. Breslow's condition (1981, Biometrika 68:73-84) has been found to be faulty for the consistency of the Mantel Haenszel estimator for large sparse tables. This finding clarifies the confusion over this point in the vast literature. New methodology has been developed to study two sequences of probabilities. It is applied to study the black-white differences in birth weight specific mortality curves. It is also extended to study the Hardy-Weinberg Law across strata.

Significance to Biomedical Research and the Program of the Institute: Odds ratio and standardized risk difference parameters are commonly used parameters in many biomedical research and epidemiological studies. One example is the Better Babies Project in which the P.I. has participated. This study will enable better understanding and more efficient use of these parameters in substantive research programs. Testing procedures so developed provide more power in comparing black-white differences in birth weight specific mortality curves.

Proposed Course of Project: Continuation of development of statistical methodology is planned. Statistical properties are to be investigated. Applications to substantive fields such as genetics will be further examined.

Publications:

Troendle JF, Yu KF. A note on testing the Hardy-Weinberg Law across strata. Ann Hum Gen 1994;58:397-402.

Jannarone RJ, Ma K, Yu KF, Gorman JW. Extended conjunctoid theory and implementation: A general model for machine cognition based on categorical data. Prog Neural Net 1995;3:361-425.

Yu KF. A necessary and sufficient condition for the strong consistency of a family of estimators of the common odds ratio. Can J Stat 1995;23:215-25.

Yu KF. A simple comparison of two sequences of probabilities. J Plan Inference 1995. (In Press)

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00856-04 BB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Statistical Methods for a Mixture of Subpopulations

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: K.F. Yu Mathematical Statistician BMSB, DESPR, NICHD

Others: A.A. Herman Visiting Scientist EB, DESPR, NICHD
H.J. Hoffman Chief EB, OD, NIDCD

COOPERATING UNITS (if any)

Norway National Institute of Public Health (L.S. Bakkeiteig); National Center for Health Statistics (C.J. Krulewitch); Chung Yuan Christian University (M.S. Yang)

LAB/BRANCH

Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human ☐ (b) Human ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This study will examine the intrinsic difficulty of estimating subpopulation characteristics when the identities of the observations are not completely known. Hard clustering and fuzzy clustering techniques to classify observations into subgroups are investigated. This study will develop estimation methodologies for the subpopulation characteristics. The statistical properties of the new methodologies will be studied. The new methodologies will be compared with the existing methodologies designed to remedy the misclassification problem. The new methodologies will alleviate some of the intrinsic difficulties for a heterogeneous mixture of subpopulations. Applications will be made to study the issues concerning biological heterogeneity and measurement error in the birth weight, gestational age and perinatal mortality. This study will also be applied to the investigation of intrauterine growth and pregnancy outcome.

Project Description:

Objectives: To develop statistical methods for studying subpopulation characteristics in a heterogeneous mixture of subpopulations.

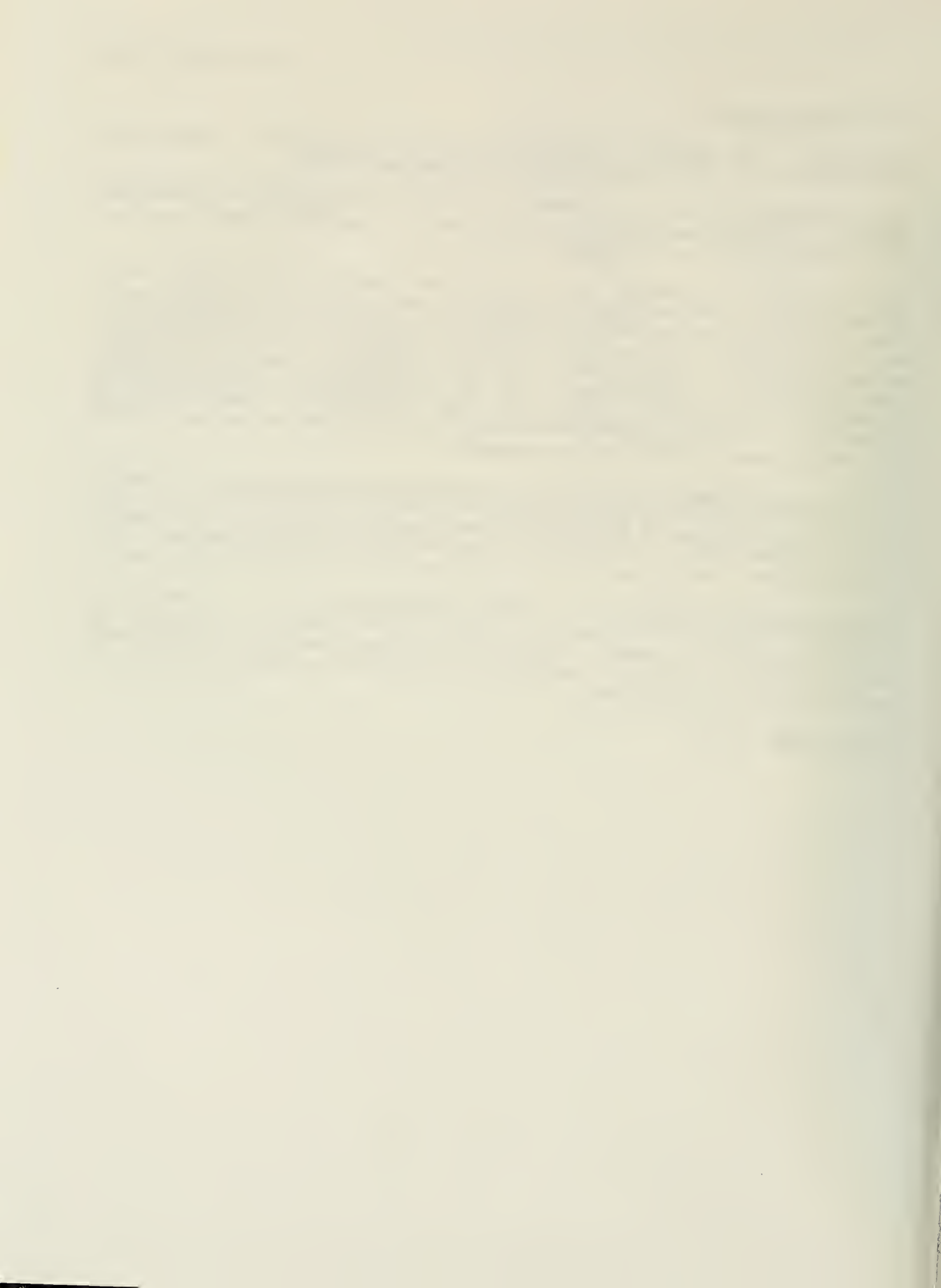
Methods Employed: Classical and modern statistical and probabilistic methods are employed. Computer technology is used as a tool for computation and simulation. New methodology is also developed.

Major Findings: Estimation of subpopulation characteristics after a hard clustering of unidentified data into subpopulations is biased and inconsistent as long as there is a chance of misclassification. A notion of fuzzy partition of the data is introduced. It has been established analytically that the developed methodologies of estimation after fuzzy classification is unbiased under some general conditions. The variance of the new methodologies is also analytically established. Simulation studies have been conducted to compare the new methodologies and other existing methodologies and show that the new ones perform much better than the others in many measures.

Significance to Biomedical Research and the Program of the Institute: Estimation after fuzzy clustering has been applied to estimate the perinatal mortality rates for the subpopulations of intrauterine growth retarded and the non-intrauterine growth retarded infants. It can also be used for other pregnancy outcomes. This methodology has also high potential for application in other substantive studies.

Proposed Course of Project: Continuation of development of this methodology is proposed. Complete investigation of the analytic results as suggested by simulation studies is planned. Application to intrauterine growth and pregnancy outcome is to be investigated. Application to study birth weight distribution and gestational age is also planned.

Publications: None



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00857-04 BB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Methodological Research in Mathematical Statistics and Biostatistics

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Y.J. Lee Chief BMSB, DESPR, NICHD

Others: J. Troendle Senior Staff Fellow BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

Hankook University of Foreign Studies (T. Park), Mayo Clinic (S. Jung), ATG (H. O'Grady)

LAB/BRANCH

Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

2.0

PROFESSIONAL:

2.0

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human ☐ (b) Human ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Methodological research in statistics is motivated often by interest in methodological/theoretical statistical questions. Consulting and collaborative projects also motivate the research because existing methods and theories may be inadequate to handle their data analysis or study design.

Dr. Lee has completed a paper on a two-sample nonparametric test method for missing observations and presented as an invited paper at the August Multivariate Statistical Inference Conference. Dr. Lee has presented a number of invited statistical talks on clinical trial methodologies in South Korea and China. Drs. Lee and Park are working on a paper on incomplete data problems in clinical trials, and on a problem to improve coefficient estimates for repeated measure data. Dr. Troendle has been working on multivariate permutational problems as well as on combining multiple testing and global testing methods. Dr. Park has been working on multivariate regression methods. Drs. Lee and Jung have completed a paper on generalizing the logistic regression to be applied to survival data, and the paper has been accepted for publication in the Journal of the American Statistical Association, subject to revision. Drs. Lee and O'Grady have been working on sample size problems for the two sample Wilcoxon test for ordinal categorical data. They are also working on nonparametric methods for the data from cross-over designs.

Project Description:

Objectives: To develop statistical methods and theories for the analysis of complex biomedical data and for the design of new biomedical studies.

Methods Employed: Both mathematical methods and computer simulation evaluation methods are applied to develop and evaluate new and existing statistical methods for design, implementation and evaluation of biomedical data analysis.

Major Findings: Dr. Lee has developed a nonparametric method for missing data and submitted a manuscript for publication. Drs. Lee and Park have completed simulation evaluations of statistical methods for two sample data where some are incomplete because of premature death of study subjects. Simulation evaluations show that the method assigning a worst possible score to the incomplete data is not statistically satisfactory. A paper has been submitted for publication.

The logistic regression method is easy to understand and flexible to model the t-year survival probability. But when some data are censored, one cannot regress the t-year survival probability against covariates. Drs. Lee and Jung have proposed a maximum conditional likelihood approach to regressing the t-year survival probability. The method can be applied to estimating the survival distribution for given covariates. A paper has been submitted and accepted for publication in the Journal of American Statistical Association.

Medical outcomes are often measured by ordered categorical scores such as severity score, performance status, etc. The two-sample discrete Wilcoxon test is broadly applied to analyzing the ordered categorical data. When designing studies with such outcome data, however, there is no satisfactory method for determining the sample size. Drs. Lee and O'Grady have undertaken a methodological investigation for determining the sample size for the two sample discrete Wilcoxon test. Simulation evaluations show that the power of the test is minimized under the slipped configuration of probability distributions. They are now working on an analytical proof of their observation from the simulation evaluations.

Dr. Troendle has been working on constructing a permutational step-up testing procedure for adjusting p values for multiple endpoints. Step-up tests are more powerful than step-down tests in certain situations. Although the algorithm derived for step-up adjusted p values is more computational than the existing step-down algorithm, some modifications have been made to reduce the computation. A paper has been submitted for publication.

A simulation study of the properties of step-down permutation tests has been completed by Dr. Troendle and Clifford Blair. It was found that the step-down tests are particularly powerful when many endpoints are involved, the data are significantly correlated, or distributional assumptions are questionable. A paper has been accepted for publication.

When multiple endpoints are involved, closed testing algorithms in conjunction with global tests have been proposed for use in identifying significant outcomes. An alternative is to use a stepwise assessment of the endpoints, where at each step an adjustment is made for the endpoint left. Dr. Troendle has completed a comparative study of some new and some existing methods for small sample sizes.

Dr. Troendle has discovered the most powerful permutation test among all similar tests against a simple normal alternative in the multivariate two sample location shift problems. Unfortunately, no more powerful test exists for composite

hypotheses. A simple approximation yields a new test which is applicable to composite one sided tests. The new test has been compared to Hotelling's T^2 permutation test and to Boyett and Shuster's maximal t test. A paper has been submitted for publication.

Drs. Troendle, Yu and Herman have been working on standardization systems for birth weight and gestational age when the outcome measure is infant mortality. The analysis compares an ad hoc method of grouping infants into simple categories of birth weight (Wilcox, 1979) to a new procedure which extends the Wilcox method to incorporate gestational age while removing much of the ad hoc methods employed in that work. The method provides a decomposition of the infant mortality for a population into two sources: that expected for a standard population with a bivariate Gaussian distribution of birth weight-gestational age pairs centered at the population means and having the same birth weight-gestational age specific mortality, and the additional mortality obtained because of the actual skewed distribution of births. Applied to U.S. data, the new method shows a contrast to the conclusions of Wilcox and Russell (1986).

Dr. Park proposed a general class of multivariate regression models which can handle both discrete and continuous repeated measurements. The proposed model is based on the seemingly unrelated regression models. The regression parameters of the model were consistently estimated using the two-stage least squares method. A paper has been published.

Drs. Park and Brown consider categorical data with binary responses subject to non-ignorable non-responses. When fitting log-linear models for these data, they showed that the ML estimation method may often yield estimators with infinity or -infinity. They proposed a constrained ML estimation by restricting the parameter space of models. With a reasonable choice of boundary constraints, they show that the proposed estimators perform better than the ML estimators. A paper has been submitted for publication.

The generalizing estimating equations approach is widely applied to analyze repeated categorical data. In this approach, the correlations among the observations from the same subject has been estimated using the Pearson residuals. For discrete distributions, these Pearson residuals are not normally distributed. Drs. Park, Davis and Li have considered using different residuals: Anscombe and deviance which are more normally distributed than the Pearson residuals. They compared three residuals using the simulation studies. The results have been summarized into a paper which has been submitted for publication.

Drs. Lee and Park continue to work on obtaining well-defined residuals which are more normally distributed than Pearson, Anscombe or Deviance residuals. Drs. Lee and Park have considered Box-Cox types of transformations and have undertaken simulation studies to investigate their properties.

In repeated measures studies, the main interest often lies in comparing groups effects. The comparison of group effects can usually be performed by testing the equality of group means. Sometimes groups are formulated in an order relation. Dr. Park proposed a test procedure for testing the equality of group means in this situation and showed that the proposed method is more powerful than the conventional one. A paper has been submitted for publication.

Significance to Biomedical Research and the Program of the Institute: We will be better able to analyze biomedical data and design studies.

Proposed Course of Project: Continue to explore and develop statistical methods applicable to biomedical data and projects.

Publications:

Troendle J. A stepwise resampling method of multiple hypothesis testing. J Am Stat Assoc 1995;90:370-8.

Troendle J, Yu K. A note on testing the Hardy-Weinberg Law across strata. Ann of Hum Gen 1994;58:397-402.

Jung SH, Lee YJ. Logistic regression of t-year survival probability: Maximum conditional likelihood method. J Am Stat Assoc, in press.

Blair RC, Troendle JF, Beck RW. Control of family wise errors in multiple endpoint assessments via stepwise permutation tests. Stat Med, in press.

Park T. Multivariate regression models for discrete and continuous repeated measurements. Com Stat Theory Meth, in press.

Park T, Brown MB. Methods for categorical data with nonignorable nonresponse. J Am Stat Assoc 1994;89:44-52.

The first part of the paper discusses the importance of understanding the underlying mechanisms of the observed phenomena. This is followed by a detailed analysis of the data, which shows that the results are consistent with the theoretical predictions. The second part of the paper focuses on the experimental setup and the results obtained from the experiments. The third part discusses the implications of the findings and the potential applications of the research. The paper concludes with a summary of the main findings and a list of references.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00859-04 BB

PERIOD COVERED

October 1, 1994 to September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Meta-Analytic Methods

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: Rebecca DerSimonian

Mathematical Statistician

BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Biometry and Mathematical Statistics Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.60

PROFESSIONAL:

0.60

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human

☐ (b) Human

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project consists of four components which continue from last year: The first compares a fixed and a random effects model for combining data from a series of clinical trials. The study evaluates the performance of each method under various heterogeneity assumptions and with several scales of measurement, including the risk difference and the odds ratio as measures of effect.

The second develops and compares parametric and non-parametric tests to assess the assumption of homogeneity of effects in data from a series of trials. This component considers data from Gaussian, binomial, and Poisson sampling distributions.

The third considers meta-analytic methods for combining the evidence from a series of HIV seroprevalence studies to estimate HIV prevalence in a target population. Sentinel studies are typically investigations of incompletely defined cohorts which are convenient to survey but are often based on non-probability samples. Self-selection and similar issues inherent in sentinel studies makes generalizing results from a single study to a target population problematic. This research assesses the use of formal meta-analytic methods for HIV prevalence estimation in a target population by incorporating information from several HIV sentinel seroprevalence studies. Preliminary results from this component is to appear in Annals of Epidemiology (in press).

The fourth component addresses issues that pertain to the use of meta-analysis in the design and monitoring of clinical trials. This research evaluates the role of formal incorporation of external evidence summarized from a meta-analysis of previous or concurrent results into sample size considerations and stopping rules during the conduct of a clinical trial. Preliminary results from this component is to appear in Statistics in Medicine.

Project Description:

Objectives: To develop statistical methods for combining data from a series of clinical trials and HIV seroprevalence sentinel studies.

Methods Employed: For meta-analysis of clinical trials, this project considers fixed and random effects as well as Bayes models for meta-analysis of data under various heterogeneity assumptions and several scales of measurement, including the risk difference and the odds ratio as measures of effect. Both parametric and non-parametric methods are employed for assessing homogeneity of effects. The role of meta-analysis in the design and monitoring of a new clinical trial is also considered.

For meta-analysis of HIV seroprevalence studies to estimate prevalence in the target population, this project considers empirical Bayes methods to incorporate information from related sentinel studies. This research characterizes the special epidemiologic and statistical issues inherent in sentinel studies for HIV prevalence estimation and develops methodology that addresses them when incorporating the information from several such studies.

Major Findings: For meta-analysis of clinical trials, several tests of heterogeneity for binomial, Gaussian, and Poisson sampling distribution are developed and compared. Fixed and random effects, as well as Bayes models, are evaluated and compared under various heterogeneity assumptions. Furthermore, the impact of using meta-analytical results in the design and monitoring of the NICHD trial on preeclampsia is evaluated (to appear in Statistics in Medicine).

For meta-analysis of seroprevalence studies, we find that prevalence in an individual HIV sentinel serosurvey is time-averaged and vulnerable to several time-dependent biases and that self-selection and laboratory methods are additional likely sources of bias. Incorporating the information from several such HIV sentinel seroprevalence studies mitigates the possible impact of these biases inherent in the individual studies (to appear in Annals of Epidemiology).

Significance to Biomedical Research and the Program of the Institute: The statistical methods developed in this project are applicable to many data sets relevant to the Institute where the evidence on a particular topic is conflicting and/or the sample sizes in the available studies are too small to yield unequivocal results. An example of the applicability of the methods to relevant problems in the Institute is the workshop organized by the Division on "The Role of Meta-Analysis in the Design and Monitoring of Clinical Trials." Being directly relevant to this topic, preliminary results from this project were presented at the workshop, which was prompted by concerns raised at the Data Safety and Monitoring Board of the large multi-center trial conducted by the Institute on "Calcium Supplementation for the Prevention of Preeclampsia."

Proposed Course of Project: Continue to explore and develop the methodology for tests of homogeneity, particularly the non-parametric ones, to further evaluate the role of meta-analysis in planning a new clinical trial, and to develop methods for combining data from HIV sentinel studies, especially when covariate information may be missing in some studies.

Publications:

Strickler H, Hoover DR, DerSimonian R. Problems in interpreting HIV sentinel seroprevalence studies. *Ann Epidemiol* 1995. (In Press)

DerSimonian R. Meta-analysis in the design and monitoring of clinical trials.
Stat Med 1996. (In Press)



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01802-05 CSB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Data Coordinating Center for the NICHD Study of Early Child Care

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: B.K. Knoke Computer Specialist (10/94-5/95) CSB, DESPR, NICHD
A.C. Trumble Computer Specialist (6/95-9/95) CSB, DESPR, NICHD

COOPERATING UNITS (if any)

CRMC, NICHD/NIH

LAB/BRANCH

Computer Sciences Branch, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, MD 20892

TOTAL STAFF YEARS:

1.6

PROFESSIONAL:

.85

OTHER:

.75

CHECK APPROPRIATE BOX(ES)

☒ (a) Human ☐ (b) Human ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This study is a 10-site cooperative agreement to examine the effects of non-maternal care, especially during the first year of life. It focuses on the social and intellectual development of children in an ecological framework which takes into account the complex interactions of non-maternal care experiences with home and family conditions, parenting practices, and child characteristics. Over 1200 families were enrolled in the study during 1991. These families will participate in the study for 36 months.

The Data Coordinating Center (DCC) is responsible for data management and protocol monitoring. It serves as the focal point for receipt of collected data and for data processing.

The DCC is currently processing 24 and 36 month data. The data are entered into computer files, edited and summarized for monitoring purposes. Routine edit reports are being distributed to the sites regularly. Project monitoring reports are distributed to the Steering Committee quarterly.

Project Description

Objectives: To examine the effects of non-maternal care, especially during the first year of life on the social and intellectual development of young children. The Data Coordinating Center is located in the Computer Sciences Branch of the Division of Epidemiology, Statistics and Prevention Research. The objective of the Data Coordinating Center is to assure that the protocol is uniformly administered at all sites. This includes the preparation of Manuals of Operations; management of monitoring subject recruitment; design of data collection instruments, data entry methods and data base management systems; and generation of statistical reports.

Methods Employed: At each of the ten sites participating in the study, approximately 120 infants and their parents were enrolled in this study shortly after the infant's birth and will be followed until the infant is 3 years old. Over the 36 months of participation, the infant will be visited and observed in the home, in child care, and in a laboratory setting. The sample was selected using a weighted method with the aim of recruiting a minimum of 10% minority, 10% low education and 10% single parent families. In addition, the sample was weighted to contain 60% mothers planning to return to work full time, 20% planning to work part-time and 20% not planning to return to work.

Through close monitoring of the sample recruitment, a sample has been selected which closely represents the goal. This study is being conducted in collaboration with the Center for Research for Mothers and Children.

Major Findings: All findings are preliminary at this time.

Significance to Biomedical Research and the Program of the Institute: This project will help determine the extent and type of non-maternal care used in this country and the effects that different patterns of care have on the development of children in the first three years of life.

Proposed Course: The data collection for phase I is almost complete. The Data Coordinating Center for phase II will be carried out under a grant.

Publications:

Friedman S, The NICHD Early Child Care Network. Child care and child development: The NICHD study of early child care. In: Friedman S, Haywood HC, eds. Developmental follow-up: concepts, domains and methods. San Diego: Academic Press, 1994;337-96.

The first part of the paper is devoted to a general
 discussion of the problem. It is shown that the
 problem is equivalent to the problem of finding
 the minimum of a certain function. This function
 is then expressed in terms of the elements of the
 matrix. The next part of the paper is devoted to
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 the equations for the elements of the matrix. The
 final part of the paper is devoted to the solution
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 matrix.

DIVISION OF EPIDEMIOLOGY, STATISTICS AND PREVENTION RESEARCH

Computer Sciences Branch

Bijur PE, Trumble AC, Harel Y, Overpeck MD, Jones HD, Scheidt PC. Sports and recreation injuries in U.S. children and youth. Arch Pediatr Adolesc Med, in press.

Friedman S, The NICHD Early Child Care Network. Child care and child development: The NICHD study of early child care. In: Friedman S, Haywood HC, eds. Developmental follow up: concepts, domains and methods. San Diego: Academic Press, 1994;337-96.

Harel Y, Overpeck MD, Jones, Scheidt PC, Bijur PE, Trumble AC. The quality of proxy-responder data in NCHS Surveys. [Letter to Editor] Am J Public Health 1995;85:591.

Overpeck MD, Trumble AC, Brenner R. Population-based surveys as sources of U.S. injury data and special methodological problems. Proceedings of the International Collaborative Effort on Injury. Hyattsville, MD: National Center for Health Statistics, 1995; DHHS publicationno. (PHS)95-1252;12-17.

Scheidt PC, Harel Y, Trumble AC, Jones DH, Overpeck MD, Bijur PE. Epidemiology of non-fatal injuries in children and youth. Am J Public Health 1995;85:932-38.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 HD 01703-06 ESPR
PERIOD COVERED October 1, 1994 through September 30, 1995		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Study of Pregnancy Outcome, Maternal Death and Child Health in Pakistan		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) PI: H.W. Berendes Director DESPR, NICHD		
COOPERATING UNITS (if any) Department of Community Health, Aga Khan University (Drs. Joseph McCormick, Fariyal Fikree, Farid Midhet, and Mehtab Karim), Special Program for Research, Reproduction and Family Planning of the WHO (Dr. Jose Villar)		
LAB/BRANCH Office of the Director, DESPR		
SECTION		
INSTITUTE AND LOCATION NICHD, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS: 0.2	PROFESSIONAL: 0.1	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) Human <input type="checkbox"/> (b) Human <input type="checkbox"/> (c) Neither <input checked="" type="checkbox"/> (a1) Minors <input checked="" type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) <p>This project has three components.</p> <p>The Maternal and Infant Mortality Survey (MIMS) will provide estimates of maternal and infant mortality rates from population based samples in Karachi and the four provinces of Pakistan. In addition, the Survey will provide information on causes of maternal and infant deaths and demographic and other determinants of maternal and infant mortality.</p> <p>The Pregnancy Outcome Study (POS) is a cross sectional and prospective study of pregnant women from selected katchi abadies to identify determinants of poor birth outcome in this population.</p> <p>The Child Survival Study (CSS) is a follow up study of live born children from POS through two years of age to assess mortality, morbidity and physical and developmental measures.</p> <p>A new component is the development of an intervention to reduce infant mortality and morbidity by training all levels of care providers (dais, lady health visitors, midwives, physicians) to recognize timely high risk conditions during pregnancy or at delivery, set up a referral and transportation system and get high risk women timely referral to a tertiary medical facility.</p>		

Project Description

Objectives: Based upon the findings on the Maternal and Infant Mortality Surveys an intervention is being developed in Karachi to reduce maternal and infant mortality. Specifically what is proposed is to conduct a study to evaluate the effectiveness of an intervention strategy which will consist of training, monitoring and supervision of providers, setting up a referral and transport system to tertiary care providers of high risk women and women with complications around the time of labor and delivery and information, education and communication campaign to increase awareness at the community level.

Methods Employed: The Maternal and Infant Mortality Survey (MIMS) is a two step survey. The first step consists of an enumeration of household composition over the last five years and identification of any deaths occurring during that period. The second step is a detailed interview (verbal autopsy) of household members to identify pregnancy related deaths, to obtain information about possible cause of maternal deaths, as well as of infant deaths. The survey in Karachi will be based upon sites which are currently part of a primary health care network developed by the Department of Community Health of the Aga Khan University and will include approximately 10,000 households. There will additionally be surveys done in each of the four provinces, that is, Baluchistan, Sindh, Northwest Frontier Territory, and the Punjab. Sample sizes for the provincial surveys will vary between 10-20,000 households depending on availability of funds. Cluster samples will be taken from select sites of each province.

POS will identify all pregnant women at a given time in the katchi abadies which are part of the primary health care system of the Aga Khan University Department of Community Health and follow them to delivery to identify determinants of poor birth outcome. Information is collected through questionnaire and by physical examination. Since most women deliver at home attended by traditional birth attendants, a notification system is in place notifying the project about any births within two days to obtain birth weight as well as modified Dubowitz evaluation to differentiate preterm births from intrauterine growth retardation among low birth weight children. The Pregnancy Outcome Study is completed.

CSS is a prospective follow up of live births from POS to age two to assess mortality and morbidity and determinants of physical growth and development. The follow up will include interviews, physical examination, measurements of physical growth, as well as behavioral assessments. The Child Survival Study is completed.

It is proposed to implement an intervention in a section of Karachi called Korangi consisting of ten contiguous wards with a population of approximately 500,000. As controls, another site is chosen nearby of similar size and similar population characteristics. Both intervention and control sites will be subjected to a pre- and post-intervention survey to obtain information on current maternal and infant mortality rates and complications around the time of pregnancy.

Major Findings: The MIMS Surveys have been completed now in Karachi, four sites in Baluchistan and two sites in the Northwest Frontier Province which in total include approximately 36,000 households. While the overall maternal mortality rate is high in all sites, there is considerable variation between sites, the lowest rate is in Karachi and was 2.7/1000 live births and the highest in parts of Baluchistan of about 12/1000. Infant mortality rates vary accordingly, also from a low of about 75/1000 to a high of 210/1000 live births. This range represents different katchi abadies within the city of Karachi. During the past year, surveys in the Northwest Territory have been completed and analyses are in progress. A new survey of MIMS in Sindh Province has been initiated.

Considerable progress has been made in the analysis of data from the Pregnancy Outcome Study. Current analyses deal with risk factors related to intrauterine growth retardation which is a major risk factor in developing countries and accounts for the major fraction of low birth weight births in developing countries. Analysis of the data suggests that major risk factors include socioeconomic conditions, including housing construction, paternal and maternal education, paternal employment and the source of water supply, as well as ethnic differences apparent among the different ethnic groups in these communities which consists of Hindus, Muslims and Christians. Biological factors related to the risk of IUGR include young maternal age, primiparity and high parity, history of poor prior pregnancy outcome, a short interconception interval, low maternal height and weight, as well as a low mid-arm circumference and low skinfold thickness reflecting maternal nutritional status as well as a non-vegetarian diet during pregnancy. In addition, there was a marked increase in risk due to consanguinity.

In a multi-variate analysis, remaining risk factors included the source of water supply, maternal education and paternal employment, primiparity and grand multiparity, consanguinity, less than one year interval between birth to conception, low maternal height and weight and also non-vegetarian diet. These findings suggest possible intervention especially related to improved nutrition but also raising the status of women by mandating primary education of all school age girls.

The intervention is currently under development and will begin in 1996.

Significance to Biomedical Research and the Program of the Institute: The identification of determinants of maternal and infant mortality is a major research interest of the Institute and the findings of this project have specific public health importance to the country of Pakistan and other countries who are in a similar stage of development. A conference was held in the spring of 1994 to present the findings from the Maternal and Infant Mortality Surveys to an audience consisting of representatives of Ministries of Health, the Provincial Government, leading academicians and representatives of international health agencies for the purpose of acquainting this audience to the findings from the surveys but also as a means of developing possible interventions to address the high maternal and infant mortality. The World Bank participated in the planning for this conference and underwrote some of the costs. The proceedings of the conference are in preparation for publication.

The interventions to reduce maternal and infant mortality in developing countries is clearly of major public health significance and of interest to this Institute.

Proposed Course: The intervention is currently under development with active participation of the Director of the Division. Funding is provided by the World Bank and by AID with some funding expected also from UNICEF in Pakistan. The Director, DESPR will serve as a senior technical advisor in this project with active participation in its design and implementation as well as the analysis of the data.

Publications:

Fikree F, Berendes H. Risk factors for term intrauterine growth retardation: A community-based study in Karachi. Bull WHO 1994;72(4):581-7.

Fikree FF, Gray RH, Berendes HW, Karim MS. A community-based nested case-control study of maternal mortality. Int J Gyn Obstet 1994;47:247-55.

Fikree FF, Berendes HW, Villar J. A rapid nutritional evaluation of pregnant women in low socioeconomic settlements of Karachi, Pakistan. J Pakistan Med Assoc, in press.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01704-05 ESPR

PERIOD COVERED

October 1, 1994 through October 31, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Stunting Among Bedouin Arab Children in the Negev, Israel

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: H.W. Berendes

Director

DESPR, NICHD

COOPERATING UNITS (if any)

Cancer Prevention Studies Branch, DCPC, NCI, NIH (M.R. Forman); Ben Gurion University of the Negev, Beer Sheva, Israel (G. Hundt)

LAB/BRANCH

Office of the Director, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.05

PROFESSIONAL:

0.05

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human

☐ (b) Human

☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project has been completed.

Z01 HD 01705-02 E5PR

October 1, 1994 through December 1, 1994

Women's Lifestyles in Pregnancy Study, Analysis of Data

[illegible]

The David and Lucile Packard Foundation (Dr. Patricia H. Shiono); Center for Population, Columbia University (Dr. Virginia A. Rauh)

Office of the Director, DESPR

NICHD, NIH, Bethesda, Maryland 20892

0.0

☒ (a2) Interviews

The analysis of data has been completed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 01706-01 ESPR

PERIOD COVERED

November 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

A Randomized Controlled Trial for the Evaluation of a New Antenatal Care Model

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I.: H.W. Berendes Director DESPR, NICHD, NIH

COOPERATING UNITS (if any)

WHO, Geneva (J. Villar); Rosario Centre for Perinatal Research, Rosario, Argentina (J.M. Belizan); Sites in Saudi Arabia (R. Al-Mazrou); Havana (U. Farnot); South Africa (J. Moodley); Thailand (P. Lumbiganon)

LAB/BRANCH

Office of the Director, DESPR

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human ☐ (b) Human ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project consists of a randomized controlled clinical trial comparing two models of antenatal care. As background, a few years ago, the Institute sponsored with other agencies of the Public Health Service an Expert Panel on Prenatal Care which indicated that the current system of care, either in the schedule of visits as well as its contents is for the most part not based upon a careful scientific assessment. The recommendation of the Panel was to revise the schedule of prenatal care for low risk women in order to save resources which can be used for high risk women. It was also recommended that studies or trials be developed to evaluate different models of prenatal care. Prenatal care is viewed as a major determinant of infant mortality and morbidity and its careful evaluation obviously is of major research interest to the Institute.

The WHO through a technical advisory panel has developed over the last two years a new model for prenatal care for developing countries which reduces the number of visits and also alters the content of prenatal care substantially. In the revised model, the content of prenatal care will consist predominately of the recognition and diagnosis of conditions and complications of pregnancy which are known to affect the health of women or birth outcome and specific interventions to deal with these conditions once identified. The DESPR, through its Director, has been an integral part of the design of this new model of prenatal care and has served as the Chair of the Technical Advisory Panel of the WHO.

It is proposed that this trial be evaluated in four or five sites by randomizing clinics in these four or five sites. Clinics would be randomized to the current system of prenatal care or the new proposed model of prenatal care. There would be at least 12 clinics per participating center for a total of 24 clinics receiving the new model of prenatal care and 24 with the current system.

Project Description

Objectives: To conduct a randomized clinical trial comparing two models of antenatal care to establish the relative merits of each model. It is intended to test whether the proposed new model is as effective as the traditional multi-visit model with regard to maternal mortality and perinatal morbidity and mortality, and satisfaction.

Methods Employed: This study will be conducted in four or five sites. The sites consist of Rosario, Argentina; Havana, Cuba; South Africa; Thailand; and Saudi Arabia. Randomization will be by care provider units or clinics rather than by patients with provider units or clinics being assigned to the current regime of prenatal care or the new model to be tested. There will be a total of 48 units or clinic sites being randomized and the patient population enrolled in this trial will be somewhere between 24-28,000.

Major Findings: None

Significance to Biomedical Research and the Program of the Institute: To test new models of prenatal care is of major research interest and public health importance in the field of maternal and child health. Clearly if it could be shown that a revised model of prenatal care consisting of fewer visits and focusing on conditions of pregnancy known to be affecting outcome is equivalent in outcome to what is currently in place, this would result in a major saving of resources which could then be used for dealing with high risk pregnancies.

Proposed Course: The protocol for this project has been developed and the sites have been identified. The study is expected to start between October 1, 1995 and January 1, 1996 in the four or five sites. The trial will run approximately two years and the results should be available in fiscal year 1998.

The Division supports one site in Rosario through a contract with funding for the other sites provided by different donor agencies and WHO.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00331-12 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Diabetes In Early Pregnancy (DIEP)

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.L. Mills	Chief, Pediatric Epidemiology Section	EB, DESPR, NICHD
Other:	M.R. Conley	Computer Specialist	EB, DESPR, NICHD
	Y.J. Lee	Chief	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

Cornell Univ.Med.Center, NY (L.Jovanovic); Brigham and Womens Hosp., Boston, MA (L.Holmes); Northwestern Univ.Med.Center, Chicago, IL (J.L.Simpson); Univ.of Pittsburgh, Pittsburgh, PA (J.Aarons); Univ.of Washington, Seattle, WA (R.Knopp)

LAB/BRANCH

Epidemiology Branch

SECTION

Pediatric Epidemiology Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Diabetes in Early Pregnancy Project was designed 1) to examine the relationship between maternal diabetic control during organogenesis and malformations in the offspring, and to identify, if possible, a specific teratogenic factor or factors in the diabetic metabolic state; and 2) to compare early fetal loss rates in women with diabetes and in non-diabetic control subjects. We found that diabetic women who came into care before the period of organogenesis achieved better results than those who came in later; but their results were still poorer than for non-diabetic control subjects. Differences in maternal glucose levels during organogenesis did not explain the malformations in the offspring of the women who were followed throughout pregnancy. These results suggest that women who enter late (and were not under medical supervision during organogenesis) probably had poor control. This resulted in malformations due to hyperglycemia or related factors. The results from the diabetic group entering early strongly suggest that other teratogenic mechanisms were present. Regarding early fetal losses, we found that diabetic women in good metabolic control were at no higher risk for spontaneous abortion than control women; the risk of loss increased dramatically as diabetic control worsened; and the overall risk of losing a pregnancy was lower than expected, only 16%. Since these primary analyses were completed, a number of related studies have been completed (see previous reports). An analysis of the effect of metabolic control on the progression of retinopathy was published this year. Values of glycosylated protein and fructosamine have been used to study the effect of intermediate first trimester control on fetal loss. This analysis is being written up for publication. A study of infection as a risk factor for early spontaneous abortion has been completed and is about to be submitted. Assays of anti-sperm, anti-phospholipid, and anti-cardiolipid antibodies have been completed and their relationship to fetal loss is being studied. Change in insulin requirement during pregnancy is being analyzed.

Project Description: Dr. James L. Mills, Dr. Jack Lee and Ms. Mary Conley of the DESPR are involved in data editing and analysis. The principal investigators noted on form 6040 also take an active part in data analysis and reporting.

Objectives: The objectives of the DIEP were to examine the relationship between maternal diabetic control during organogenesis and malformations in the offspring; to identify, if possible, a specific teratogenic factor in diabetic metabolic state; and to compare early fetal loss rates in women with diabetes and non-diabetic control subjects. Secondary goals include investigating the metabolic milieu in early pregnancy, determining risk factors for spontaneous abortion in the non-diabetic population, and relating metabolic events in diabetic pregnancy to complications such as retinopathy in the mother and minor malformations in her offspring. A number of issues relating to metabolic events in diabetic pregnancy, malformations and retinopathy are now being examined.

Methods Employed: An innovative study design in which women were identified prior to becoming pregnant and monitored closely for early diagnosis of pregnancy was used to address these questions. Women were requested to enroll prior to becoming pregnant and in over 50% of cases subjects monitored basal body temperature in order to identify pregnancy at the earliest possible time. Data were gathered on all pregnancy losses. Malformations were identified at delivery or, when feasible, after abortion. Data from these pregnancies are now being analyzed. Repository specimens collected in anticipation of future need are now being used to answer a variety of questions.

Major Findings: The major questions have been answered. We found that diabetic women who came into care before the period of organogenesis achieved better results than those who came in later; but their results were still poorer than for non-diabetic control subjects. Differences in maternal glucose levels during organogenesis did not explain the malformations in the offspring of the women who were followed throughout pregnancy. These results suggest that women who enter late (and were not under medical supervision during organogenesis) probably had poor control. This resulted in malformations due to hyperglycemia or related factors. Data from the diabetic group entering early strongly suggest that other teratogenic mechanisms were present. Regarding early fetal losses, we found that diabetic women in good metabolic control were at no higher risk for spontaneous abortion than control women; the risk of loss increased dramatically as diabetic control worsened; and the overall risk of losing a pregnancy was lower than expected, only 16%.

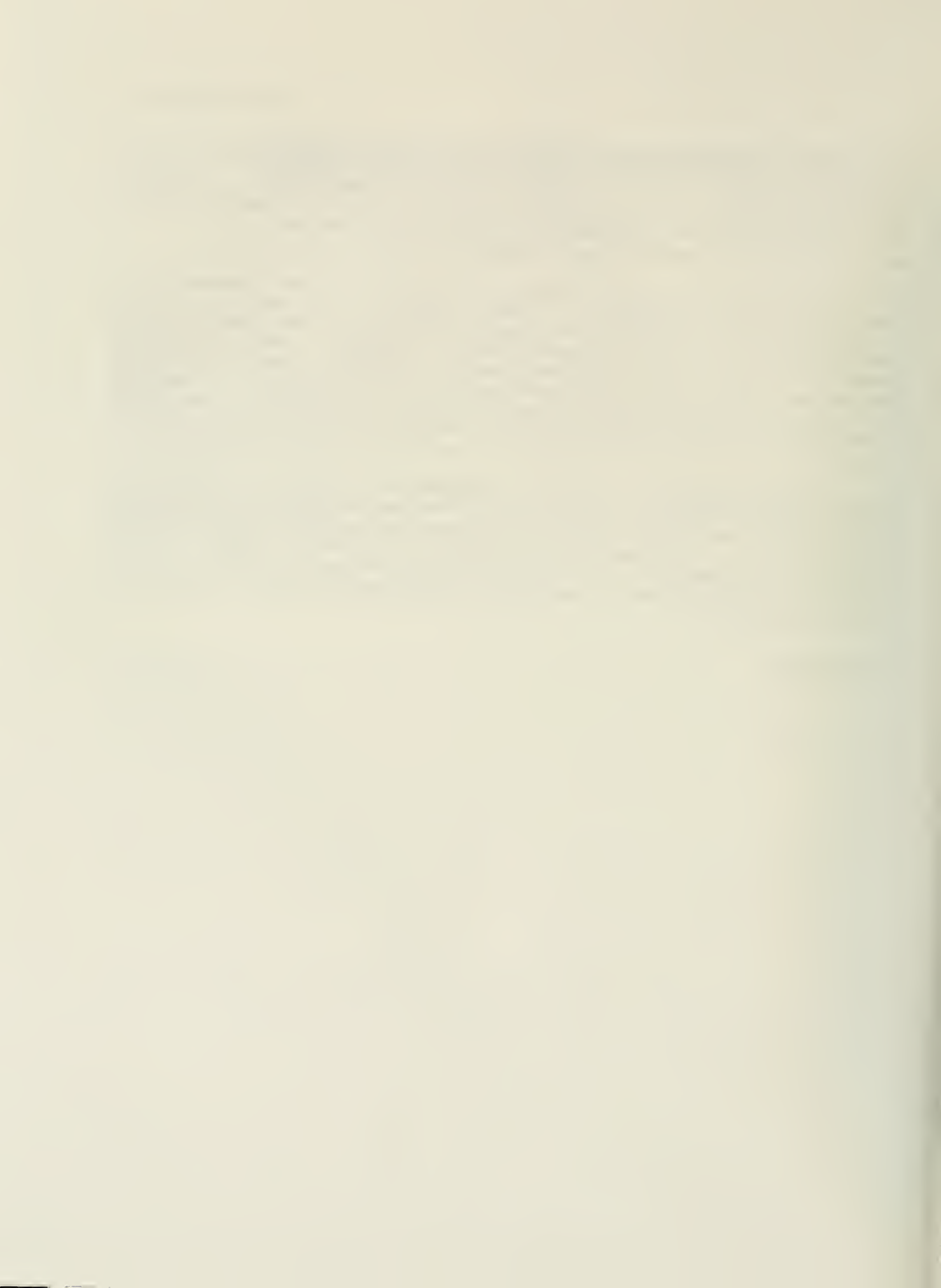
This past fiscal year we published the results of our analysis of the relationship between metabolic control and retinopathy. First trimester infection does not appear to be a risk factor for early spontaneous abortion. Autoimmune phenomena which have been implicated in second trimester loss do not appear to be significant factors in earlier loss.

Significance to Biomedical Research and the Program of the Institute: Our major findings have been that poorly controlled diabetes increases the risk of spontaneous abortion. The risk can be eliminated by good control. Congenital malformations can be reduced by good periconceptional care. However, factors other than glucose are teratogenic and elevated malformation risks are still present in women in moderately good metabolic control.

Our findings on progression of diabetic retinopathy during pregnancy have important clinical implications. Diabetic women who have moderate or worse retinopathy at the beginning of pregnancy are at high risk for progression and possible loss of vision. Such women must be monitored closely and treated promptly for signs of proliferate retinopathy. In order to reduce the risk of progression, women must be in very good control prior to conception. It was not known how good control must be to reduce the risk of complications. Our access to pre-loss blood samples which is unique is enabling us to investigate a variety of postulated risk factors not previously studied in very early pregnancy.

Proposed Course of Project: The DIEP is completing an analysis of glycosylated proteins and malformation outcomes. We will continue to look at the relationship between ketone levels and bad pregnancy outcomes, and we will examine potential patterns of minor malformations in infants with diabetic mothers. Anti-phospholipid antibodies as a risk factor for spontaneous abortion will be studied. A postulated drop in insulin requirement during the first trimester is being investigated.

Publications:



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 0334-12 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Low Birth Weight Across Generations

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: M.A. Klebanoff

Research Medical Officer

EB, DESPR, NICHD

COOPERATING UNITS (if any)

Office of the Director, DESPR, NICHD (H.W.Berendes); University of Pennsylvania (S.Katz), University of Southern California (B.Mednick)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

.25

PROFESSIONAL:

.25

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The original description of the correlation between birthweights of mothers and their infants was followed by the description of the association between large maternal birth weight and delivery of a macrosomic (>4000 gram) infant. Study of other fetal growth parameters, including length and head circumference, demonstrated that infants of low birth weight mothers were both shorter and lighter than infants of larger mothers, but that the infants were normally proportioned.

In ensuing studies, birth certificates of infants born in Tennessee between 1979 to 1984 were matched with those of their mothers, who were born in Tennessee between 1959 to 1966. Maternal and infant birth weights were again shown to be correlated. In addition, women who were themselves of low birth weight were up to 4 times as likely to have a small-for-gestational age infant as were women who weighed 4000-4499 grams at birth, but low birth weight women were less than twice as likely to have a preterm infant. A group of Swedish women, born from 1955 to 1965, was next studied. Women who themselves were small-for gestational age at birth were at increased risk of giving birth to both small-for-gestational age and preterm infants. Women who were preterm at birth were not at increased risk of either outcome.

Analysis of data from girls who were born in the 1960's as subjects in the Collaborative Perinatal Project and Danish Perinatal Study is currently underway in order to examine their reproductive histories. Small-for-gestational age, preterm and control girls have been located and interviewed. Hospital records of their deliveries have also been retrieved.



Project Description

Objectives:

- 1) Evaluation of the association between the birth weight of a mother with those of her children.
- 2) Determination of whether the relationship is due to the presence of outliers, or due to a shifting of the entire birth weight distribution in the second generation.
- 3) Separation of the second-generation effect of maternal birth weight into independent effects of maternal gestational age at delivery and maternal intrauterine growth.
- 4) Study of the effects of the perinatal experience of mothers on their subsequent reproductive and health-related outcomes.

Methods Employed: Work done prior to FY94 has addressed objectives 1 and 2. Work continuing in FY94 is directed at addressing objectives 3 and 4. Two studies of similar design are currently being analyzed. In both of these studies, reproductive-age women whose own births had been extensively documented due to their participation in a study of pregnancy were located, and their pregnancy outcomes determined. One study, done under contract by the University of Pennsylvania (subcontracting to Brown University), has traced approximately 120 women who were themselves born preterm, 160 who were small-for-gestational age (SGA), and 350 term, appropriately grown controls. These women were originally subjects in the Philadelphia and Providence cohorts of the Collaborative Perinatal Project. Reproductive histories have been obtained from these women, and medical records from their deliveries have been abstracted. A second contract, awarded to the University of Southern California (subcontracting to the Psykologisk Institut in Copenhagen), has traced approximately 158 parous women who were themselves preterm, 160 who were SGA, 37 who were both preterm and SGA, and 943 parous term controls. These women were originally subjects in the Danish Perinatal Study (1959-61). The extensive record linkage system in Denmark has enabled the collection of data from nearly 100% of all second-generation pregnancies. In addition, the Danish record linkage system has enabled the collection of data on paternal birth weight and (through military draft records) paternal adult height and weight.

Major Findings: The original description of the correlation between birthweights of mothers and their infants was followed by the description of the association between large maternal birth weight and delivery of a macrosomic (>4000 gram) infant. Study of other fetal growth parameters, including length and head circumference, demonstrated that infants of low birth weight mothers were both shorter and lighter than infants of larger mothers, but that the infants were normally proportioned.

In ensuing studies, birth certificates of infants born in Tennessee between 1979 to 1984 were matched with those of their mothers, who were born in Tennessee between 1959 to 1966. Maternal and infant birth weights were again shown to be correlated. In addition, women who were themselves of low birth weight were up to 4 times as likely to have a small-for-gestational age infant as were women who weighed 4000-4499 grams at birth, but low birth weight women were less than twice as likely to have a preterm infant. A group of Swedish women, born from 1955 to 1965, was next studied. Women who themselves were small-for-gestational age at birth were at increased risk of giving birth to both small-for-gestational age and preterm infants. Women who were preterm at birth were not at increased risk of either outcome.

Work in FY95 has yielded several findings. In the Danish study, the women had 2046 singleton infants, 24.7% of infants born to SGA mothers were SGA; in comparison, 11.4% of infants born to appropriately-grown mothers were SGA ($p < 0.001$). Women who were themselves preterm at birth had a 10.5% risk of giving birth to a preterm infant in any given pregnancy, compared to 6.6% for women born at term ($p = 0.04$). SGA mothers were not at significantly increased risk of giving birth to a preterm infant, and preterm mothers were not at significantly increased risk of giving birth to a SGA infant. Adjustment for confounding variables did not change the results. In addition, the association between maternal and infant SGA varied depending on the height of the grandmother. When the grandmother was tall, maternal SGA was not a risk factor for infant SGA. However, when the grandmother was short, maternal SGA added significantly to the infant's risk of being SGA. Additional analyses from this study addressed the antecedents and effects of uncertainty of the gestational age estimate.

Preliminary analysis of the data from Philadelphia and Providence indicates that the 627 interviewed women had 1131 children. Women who were themselves preterm at birth were not at increased risk of giving birth to a low birth weight infant (relative risk 1.1), but that women who were small-for-gestational age at birth were at significantly increased risk of giving birth to a low birth weight infant (relative risk 1.7, $p < 0.05$). Data regarding infant gestational age are currently being edited.

Significance to Biomedical Research and the Program of the Institute: This project suggests that the pathogenesis of low birth weight begins even before the birth of the mother. This implies that future research on prevention and management of at risk pregnancies should take into account the intrauterine and perinatal experience of a mother. It also implies that changes in the incidence of preterm delivery and intrauterine growth retardation may take several generations to accomplish. Further research is needed to determine the relative contribution of genetic effects versus environmental factors (e.g. smoking) passed across generations on low birth weight.

Proposed Course of Project: Data analysis is ongoing and several manuscripts are being drafted.

The Tennessee data were published in the Journal of Pediatrics. The results from Sweden have been published in Pediatrics. A paper describing the Danish study methods was published in Paediatric and Perinatal Epidemiology.

Further results of this project will be published in peer-reviewed journals.

Publications: Previously listed.

Presentations: Preterm and small-for-gestational age birth across generations in U.S. whites and blacks. American Public Health Association, 1992.

Uncertain gestational age - a source of bias in epidemiologic studies. Society for Pediatric Epidemiologic Research, 1995.

SGA and preterm birth across generations. Society for Pediatric Epidemiologic Research, 1995.

Contract Report:

This project has been supported by the University of Pennsylvania, under NICHD contract Number N01-HD-7-2909, and by the University of Southern California, under NICHD contract Number N01-HD-7-2902. These contracts, both entitled "Preterm and Small for Gestational Age Birth Across Generations," were initiated on 3-1-87 and 5-1-87, respectively, and terminated during FY92. The main objectives of these contracts have been to locate, interview and abstract the obstetrical records of a group of women who were themselves preterm, small for gestational age or normal-sized at birth. The methods employed included interviews with human subjects, and medical record abstraction. During the current fiscal year, interviewing was completed, medical record abstractions have been completed, and data processing and analysis are ongoing. The Project Officer has been extensively involved in the design and execution of these contracts. He designed the sample selection procedures and had primary responsibility for designing the study forms. In addition, he closely monitored the subject location procedures and was responsible for developing solutions to aid in increasing the follow-up rate. He is also responsible for carrying out the data analysis. This contract is essential for locating and interviewing the appropriate research subjects, because such women are otherwise unavailable to NICHD researchers.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00361-09 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Child Health Supplement to the 1988 National Health Interview Survey

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: M.D. Overpeck

Epidemiologist

EB, DESPR, NICHD

Other: A. Trumble

Acting Branch Chief

CSB, DESPR, NICHD

COOPERATING UNITS (if any)

HLB, CRMC, NICHD (P.C.Scheidt); University of North Carolina at Chapel Hill (J.Kotch); Bar Ilan University (Y.Harel); Centers for Disease Control (D.Jones); Albert Einstein College of Medicine (P.Bijur)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.1

PROFESSIONAL:

1.0

OTHER:

0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

NICHD sponsored and coordinated the 1988 survey to provide population-based data on children's health. Early work focused on effects of low birth weight and maternal smoking on children's later health status. The later work has analyzed childhood injury risk factors to support effective targeting of interventions since injury is the leading cause of death for children in the U.S. This is the first study to demonstrate that non-fatal injury causes differed considerably from fatal causes, with considerable variation of risk factors by age and socioeconomic characteristics. Analysis of injuries in preschool children by child care patterns showed that use of non-guardian care did not increase risks except for the youngest children of poor, low education mothers who use multiple part-time places of care of all types (center-based, care in their own home or homes of others). Increasing hours in center-based care seemed to lower both total and serious injury risks for children ages 4-5 years. Also, older children of women who are problem drinkers, or of two parents who are both problem drinkers, are at higher injury risk. Another risk factor analysis showed that sports and recreation account for 36% of all medically attended injuries from all causes. Analysis of effects of access to medical care showed that for children without medical care coverage (health insurance or Medicaid), as much as 30% of total injuries and 40% of serious injuries may not have received medical attention compared to children with medical care coverage. The latest studies have shown that children in single adult households are at increased risk regardless of other factors such as poverty or race.

Project Description

Objectives: 1) To provide prevalence estimates of health status indicators, injuries, child care, and family structure for all U.S. children. 2) To learn the determinants of children's health and injury risks.

Methods Employed: During 1988 NCHS conducted an interview survey of a representative sample of all households in the U.S. as part of the continuous National Health Interview Survey. About 17,200 sample children were selected. Questions pertained to the child's perinatal and birth period, family structure, child care, health status and behavior. The questionnaire was developed by a cooperative effort from representatives of the NICHD, U.S. Health Resources and Services Administration, National Center for Health Statistics and Child Trends, Inc. The Epidemiology Branch of NICHD had major responsibility for coordination.

Major Findings: 1) No effect on injury risk for preschool children was found for most nonguardian child care use. However, risk of serious injury decreased with increasing hours in center-based care at ages 4-5 years. Serious injury risk increased for children <2 years old whose mothers had <12 years of education and who used part-time and multiple places of care across all types of care combined compared to using either full-time or no care.

2) Twelve-month recall of medically attended injuries is affected by severity of the injury, age and sex of the child, and the type of injury event.

3) Non-fatal injuries show a different epidemiological pattern from fatal injuries reflecting interaction of types of exposures and developmental stages.

4) Children of women who are problem drinkers have an elevated injury risk; children with two parents who are problem drinkers are at higher risk.

5) Parents of children without either health insurance or Medicaid are less likely to report medically attended injuries, regardless of injury severity. Studies based on injury reports from treatment sources could be biased when assessing the effects of exposures associated with lack of medical coverage.

6) Sports injuries account for 36% of all medically-attended injuries. Cause and nature of injury are strongly related to age.

7) Children living in single parent households are 40 percent more likely to be injured than those in two-parent households.

Significance to Biomedical Research and the Program of the Institute: This survey documents current health status and its relation to accidents, injuries, poisonings, exposure to cigarette smoke, early health conditions, family structure, child care, and use of health services and behavior. Normative ranges are established. The data enable prioritizing of targeted interventions and research for children through representative U.S. population-based estimates.

Proposed Course: Three final analyses on injury risks associated with child care and socioeconomic factors are under review for submittal or publication.

Publications:

Overpeck MD, Kotch JB. Effects of access to care on medical attention for injuries. Am J Publ Hlth 1995;85:402-4.

Overpeck MD, Trumble AC, Brenner RA. Population-based surveys as sources of U.S. injury data and special methodological problems. Proceedings of the International Collaborative Effort on Injury, Bethesda, MD, May 18-20, 1994. Hyattsville, MD: DHHS Pub. No. (PHS) 95-1252;12-7.

Scheidt PC, Harel Y, Trumble AC, Jones DH, Overpeck MD, Bijur PE. Epidemiology of non-fatal injuries in children and youth. Am J Publ Hlth 1995;85:932-8.

Harel Y, Overpeck MD, Jones DH, Schedit PC, Bijur PE, Trumble AC. The quality of proxy-respondent data in NCHS surveys. [Letter to the Editor]. Am J Publ Hlth 1995;85:591.

Overpeck MD, Moss AJ. Wease; words?: passive reporting on passive smoke. [Letter to the Editor.] MediaCritic 1994;2:104-5.

Overpeck MD, Moss AJ. Smoking studies. [Letter to the Editor.] Los Angeles Times, August 3, 1994.

Bijur PE, Trumble AC, Harel Y, Overpeck MD, Jones DH, Scheidt PC. Sports and recreation injuries in U.S. children and youth. Arch Pediatr Adolesc Med, in press.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00369-07 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Adverse Perinatal Events and Subsequent Injury-related Death

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M.A. Klebanoff

Research Medical Officer

EB, DESPR, NICHD

COOPERATING UNITS (if any)

NIMH (M. Farmer)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.05

PROFESSIONAL:

0.05

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Several published studies have indicated that children experiencing asphyxia during the perinatal period are at increased risk of subsequent adolescent suicide. However, these studies used retrospectively ascertained data and were unable to control for the adverse social conditions that often accompany perinatal difficulties.

In the first phase of this project, the names of the approximately 55,000 children who were born during 1959-66 as subjects in the Collaborative Perinatal Project were computerized. During the second phase, the computerized names and other appropriate identifying information were submitted to the National Death Index, and all subjects who have died were identified. In the third phase, the death certificates of these subjects will be obtained and cause of death recorded. Since the Collaborative Project collected extensive data about the subjects' prenatal, perinatal, and childhood histories, it will be possible to study prospectively the relationship between adverse perinatal events and subsequent risk of death, as well as cause of death.

Project Description

Objectives: The purpose of this project is to evaluate the relationship between adverse perinatal events and subsequent rates and causes of death in a population with thoroughly documented prenatal, perinatal and childhood histories.

Methods Employed: This project will utilize individuals who were born as subjects in the Collaborative Perinatal Project, a multicenter study of pregnancy, delivery and childhood that prospectively enrolled approximately 60,000 pregnancies from 1959-66. The subjects' names, which do not exist on computer files, were entered onto a computer. This task was completed in the winter of 1993. Following this, the names, along with other required identifying information, were submitted to the National Death Index, where it was possible to match them to all deaths in the United States from 1979 to the present. Results were obtained in the spring of 1994. For subjects who have died, the death certificate will be obtained and cause of death coded. Rates and causes of death will be compared to determine the effect of perinatal asphyxia on subsequent adolescent and young adult mortality.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute: This project will provide valuable additional data on the relationship between perinatal events and subsequent young adult death, and will enable the intervening role of adverse social situation to be investigated. The project will add significantly to the present state of knowledge about the long term effects of perinatal events.

Proposed Course of Project: The preparation of the data tape has been completed. The tape was submitted to the National Death Index in the winter of 1993, and results obtained in the spring of 1994. Death certificates of possible matches will be obtained using FY96 funds.

Publications: None yet

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 HD 00373-07 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Calcium Supplementation in Pregnancy for the Prevention of Preeclampsia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.J. Levine	Expert (Epidemiology)	EB, DESPR, NICHD
Others:	R. DerSimonian	Mathematical Statistician	BMSB, DESPR, NICHD
	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
	M.A. Klebanoff	Research Medical Officer	EB, DESPR, NICHD

COOPERATING UNITS (if any)

University of Alabama (J.C.Hauth); Case Western Reserve U. (P.M.Catalano); Univ. of New Mexico (L.B.Curet); Oregon Health Sci.Univ. (C.Morris); Univ. of Tennessee (B.M.Sibai); The Emmes Corp. (J.Esterlitz); Biomedical Research Inc. (J.Leaf)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

1.4

PROFESSIONAL:

1.4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The results of ten clinical trials suggest that supplemental calcium may prevent preeclampsia. Methodologic problems, however, and differences in study design limit the credibility of the results and their generalizability to other patient populations. Moreover, none of the trials has reported the outcome of systematic surveillance for urolithiasis, an important possible complication of treatment. In response to the need for a definitive evaluation of the effects of calcium supplementation, NICHD is conducting a trial at five U.S. university medical centers. Healthy nulliparous patients are randomly assigned to receive either 2 g ingested supplemental calcium daily (n=2250) or placebo (n=2250) in a double-blind study. Medication is administered beginning between 13 and 21 completed weeks of gestation and continued until the termination of pregnancy. Monitoring for the major study endpoints - pregnancy-associated hypertension and proteinuria, preeclampsia, eclampsia, and HELLP syndrome - and for urolithiasis will be systematic, standardized, and thorough. It will include measurement of blood pressure, proteinuria, and hematuria at uniformly scheduled prenatal clinic visits and surveillance for hypertension and proteinuria during labor, delivery, and the first 24 hours postpartum. CPEP should have adequate statistical power to detect a reduction of 43 percent in preeclampsia risk in the calcium group.

Project Description

Objectives: The purpose of this investigation is to evaluate the efficacy of 2 gm/day ingested supplemental calcium for reducing the incidence of important hypertensive disorders of pregnancy - preeclampsia, eclampsia, and HELLP syndrome - in healthy nulliparous women.

Methods Employed: The study is a multicenter, randomized, double-blind, placebo-controlled clinical trial. Besides nulliparity, study subjects lack known risk factors for preeclampsia. Blood pressure is monitored at prenatal clinic visits by trained nurses using mercury sphygmomanometers and cuffs of appropriate size. Proteinuria is measured by total protein excretion in 24-hour urine collections and in random urine specimens, by dipsticks and by protein/creatinine ratios.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute: Should calcium supplementation be proven beneficial, it may become a part of routine prenatal care with the purpose of reducing the risk of hypertensive disorders of pregnancy and decreasing associated maternal and perinatal morbidity and mortality.

Proposed Course of Project: The field trial was launched in June 1992, following a one-month pilot phase. During FY 1996 the follow-up of study subjects will be completed, and the database cleaned and frozen for analysis. Site visits will be conducted, and a joint meeting of the Steering Committee and the Data Safety and Monitoring Board will be convened at which the results of the study will be presented.

Publications: Previously listed.

Contract Report: The project has been supported by The Emmes Corporation, the University of Alabama, Case Western Reserve University, the University of New Mexico, Oregon Health Sciences University, the University of Tennessee, and Biomedical Research Incorporated under the following respective contract numbers: N01-HD-1-3121, N01-HD-1-3122, N01-HD-1-3125, N01-HD-1-3124, N01-HD-1-3123, N01-HD-1-3126, and N01-HD-2-3154. During FY95 \$2,373,702 has been allotted to these contracts. The major objective of the contracts is to provide support to five clinical centers, which will enroll and follow patients for the trial; to a data coordinating center, which will be responsible for all data management and analysis; and to a central repository for storage of laboratory specimens obtained from study subjects. During the current fiscal year patient enrollment has been completed. The collection of data obtained during patient follow-up continues. The above contracts are essential since NICHD does not have facilities for enrolling and monitoring pregnant women, sufficient statistical staff to maintain a data coordinating center, or the facilities and personnel necessary for operating an ultra-low temperature storage facility.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00379-06 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Data Analysis from the Vaginal Infections and Prematurity Study

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M.A. Klebanoff Research Medical Officer EB, DESPR, NICHD

COOPERATING UNITS (if any)

PAMA, CRMC, NICHD (R.Nugent); NIAID (M.F.Cotch); Research Triangle Institute (A.V.Rao); Columbia University (J.Regan)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

.3

PROFESSIONAL:

.3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unredacted type. Do not exceed the space provided.)

The Vaginal Infections and Prematurity (VIP) Study, jointly sponsored by NICHD and NIAID, is a prospective study designed to investigate the relationship between genital tract colonization with various microorganisms and the subsequent development of preterm birth. It also incorporates a clinical trial of erythromycin to prevent preterm birth among women colonized with *Ureaplasma urealyticum*, *Chlamydia trachomatis*, and group B streptococcus. In addition, the VIP collected a wealth of additional data on a variety of factors possibly linked to pregnancy outcome. This project will analyze these factors.

Analyses undertaken to date include the predictive value of vaginal Gram stain in the identification of group B streptococcus, the descriptive epidemiology of group B streptococcal carriage, the relationship between reported physical activity and preterm birth, the effect of treatment with erythromycin on pregnancy outcome among women colonized with group B streptococci, and the association between sexual intercourse during pregnancy and preterm birth among colonized with different genital microorganisms. Questions under analysis include the effect of group B streptococcal colonization on pregnancy outcome, the association between bacterial vaginosis and pregnancy outcome, and the effect of treatment with erythromycin on pregnancy outcome among women colonized with *Chlamydia trachomatis*.

Project Description

Objectives: The objective of this project is to investigate factors associated with adverse pregnancy outcome in the Vaginal Infections and Prematurity (VIP) data set.

Methods Employed: This study, sponsored by NICHD and NIAID, is a prospective study designed to investigate the relationship between genital tract colonization with various microorganisms and the subsequent development of preterm birth. It also incorporates a clinical trial of erythromycin to prevent preterm birth among women colonized with *Ureaplasma urealyticum*, *Chlamydia trachomatis*, and group B streptococcus (GBS). In addition, the VIP collected a wealth of additional data on a variety of factors possibly linked to pregnancy outcome.

Major Findings: Several analyses have been conducted to date. The utility of a cervicovaginal Gram stain as a screening tool for carriage of group B streptococcus was evaluated. The sensitivity, specificity, positive and negative predictive values were 28, 69, 17, and 81% at mid gestation, and 34, 72, 18, and 86% at delivery. The positive and negative predictive values differed little from the baseline prevalence of the organism. It was concluded that the Gram stain was not a useful screening tool for the detection of group B streptococcal carriage.

An additional analysis detailed the descriptive epidemiology of group B streptococcal colonization during pregnancy. Various factors associated with group B streptococcal carriage were described. For example, group B streptococcus was more common among older women and women of lower parity; it was less common among women currently living with their partner, and less common among current smokers. Increasing years of sexual experience resulted in a decreased risk of colonization. Group B streptococcus was present more frequently among women colonized with *Candida* species, but group B streptococcus was not more common among women colonized with *Chlamydia*, *Ureaplasma* or *Trichomonas*, compared to women not colonized with these organisms.

The analysis of the relationship between group B streptococcal carriage and pregnancy outcome is ongoing. Preliminary analyses do not demonstrate a significant association between carriage of the organism and either preterm delivery or low birth weight. Similarly, these adverse outcomes were not more common among women heavily colonized with group B streptococcus than among those lightly colonized. However, further analysis demonstrated that nearly 25% of women received a variety of antibiotics for clinical indications during the third trimester. Among women who did not receive clinically indicated antibiotics, increasing density of streptococcal colonization was associated with a significantly increasing risk of delivery before 32 weeks, as well as with a significantly increasing risk of giving birth to a low birth weight (<2500 gram) or very low birth weight (<1500 gram) infant. Results from the clinical trial of erythromycin indicate that treatment of streptococcal carriers with erythromycin does not result in a reduced occurrence of early delivery or delivery of a low birth weight infant.

A fourth analysis investigated the relationship between reported physical activity and preterm birth. Prolonged periods of standing were modestly associated with preterm birth (odds ratio for ≥ 8 hours/day of standing = 1.31). Heavy work/exercise was not associated with preterm birth (odds ratio for ≥ 4 hours/day of heavy work = 1.04). The proportion of infants born preterm did not differ among women employed in predominantly standing, active or sedentary occupations. Physical activity was not associated with gestational age-adjusted birth weight. In an updated analysis, prolonged standing was no longer associated with preterm birth.

An additional analysis evaluated the association of reported frequency of sexual intercourse at 23-26 weeks and preterm birth. Among all women, sexual intercourse was associated with a 20% reduction of preterm birth. However, the effect of sexual intercourse was dependent on the presence of certain micro-organisms. Among women who were not colonized with *Trichomonas vaginalis*, sexual intercourse was associated with a reduced risk of preterm birth, but among women who were colonized with this organism, intercourse was not associated with preterm birth. Similar results were obtained for colonization with *Mycoplasma hominis*. Conversely, *T. vaginalis* and *M. hominis* were risk factors for preterm birth only among women reporting frequent sexual intercourse.

In another analysis, the association between the presence of bacterial vaginosis and pregnancy outcome was studied. Bacterial vaginosis was present in 16% of the study women, and was associated with the delivery of a preterm-low birth weight infant (odds ratio 1.4, 95% confidence interval 1.1-1.8). Metronidazole use between study enrollment and delivery was associated with a decrease in the occurrence of preterm-low birth weight.

Significance to Biomedical Research and the Program of the Institute: Preliminary results from the clinical trial indicate that treatment of all women colonized with group B streptococcus is unlikely to reduce their risk of preterm or low birth weight delivery. It is possible, however, that treatment of the more densely colonized women might have some benefit. These analyses indicate that Gram stain is not an effective tool to screen for group B streptococcal carriage, implying that other screening methods for this important perinatal pathogen need to be devised. The results of the analysis of the relationship between activity and preterm birth have both clinical and research implications. Practical implications are that for most women with uncomplicated pregnancies, there is no need to restrict activity in the hopes of preventing preterm birth. Important research implications are that on a population basis, changes in maternal activity during pregnancy are not likely to have large effects on the rate of preterm birth, and that unmeasured differences in socioeconomic status between women reporting different amounts and types of physical activity were likely to have accounted for previous positive results on this subject.

The results of the analysis of sexual intercourse shed important new light on the role of infection in the pathogenesis of preterm birth. First, they imply that for most pregnant women, intercourse does not adversely affect the pregnancy. Second, the results suggest that when certain organisms are present in the vagina, their introduction into the cervix (as might happen during intercourse) can lead to preterm labor.

The analyses of bacterial vaginosis have identified an important, treatable risk factor for preterm birth.

Proposed Course of Project: The analysis of group B streptococcal colonization on pregnancy outcome is continuing under review.

Publications:

Klebanoff MA, Regan JA, Rao AV, Nugent RP, Blackwelder WC, Eschenbach DA, Pastorek JG, Williams S, Gibbs RS, Carey JC for the Vaginal Infections and Prematurity Study Group. Outcome of the vaginal infections and prematurity study: results of a clinical trial of erythromycin among pregnant women colonized with group B streptococci. Am J Obstet Gynecol 1995;172:1540-5.

Hillier SL, Nugent RP, Eschenbach DA, Krohn MA, Gibbs RS, Martin DH, Edelman R, Cotch MF, Pastorek J, Rao AV, McNellis D, Regan JA, Carey JC, Klebanoff MA. The association of bacterial vaginosis, bacteroides, and *Mycoplasma hominis* with preterm, low birth weight delivery. N Engl J Med, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00382-05 EB

PERIOD COVERED

October 1, 1994 through February 28, 1995

TITLE OF PROJECT (30 characters or less. Title must fit on one line between the borders.)

Cocaine and Marijuana Use During Pregnancy and Pregnancy Outcome

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M.A. Klebanoff

Research Medical Officer

EB, DESPR, NICHD

COOPERATING UNITS (if any)

Office of the Director, DESPR (H.W.Berendes); PAMA, CRMC (R.Nugent); Research Triangle Institute (M.Cotch); Packard Foundation (P.Shiono); University of Utah (D.Rollins)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

.20

PROFESSIONAL:

.20

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☒ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project is terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00383-05 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Analyses of Data from the Collaborative Perinatal Project

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: M.A. Klebanoff

Research Medical Officer

EB, DESPR, NICHD

COOPERATING UNITS (if any)

University of California at San Francisco (T.Newman); NEI (E.Chew);
University of Maryland (D.Nagey); Packard Foundation (P.Shiono)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

.15

PROFESSIONAL:

.15

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☒ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The Collaborative Perinatal Project (CPP) represents a rich data set for the investigation of a variety of topics related to maternal and child health.

Currently active areas of investigation include the descriptive epidemiology of congenital cataract, and the association between elevated maternal serum levels of alpha fetoprotein at 14-20 weeks and subsequent stillbirth.

Project Description

Objectives:

1. To determine the association between neonatal hyperbilirubinemia and neurodevelopmental abnormalities at age 7-8.
2. To state the descriptive epidemiology of strabismus and congenital cataract.
3. To determine the association of elevated maternal serum alpha fetoprotein at 14-20 weeks and subsequent stillbirth.
4. To determine whether perinatal exposure to vitamin K is associated with an increased risk of cancer during childhood.

Methods Employed: The Collaborative Perinatal Project (CPP) enrolled pregnant women entering prenatal care at 12 university hospitals across the United States from 1959 to 1966. The women were followed throughout pregnancy, labor and delivery, and their children received multiple physical, neurological and psychological exams during their first 7 to 8 years of life. Maternal serum was collected at multiple times during pregnancy. Extensive data were collected on a wide variety of obstetric and pediatric conditions.

One investigation using this dataset completed during FY94, in collaboration with Dr. Thomas Newman of the University of California at San Francisco, evaluated the association between neonatal hyperbilirubinemia and neurodevelopmental outcome at age 7-8. The CPP required that serum bilirubin be measured on every neonate according to a fixed protocol. Extensive psychologic testing was performed at age 7, as was a detailed neurologic examination. In addition, a smaller group of children underwent tests of speech and hearing function at age 8. This analysis compared the Wechsler IQ at age 7 of children with different values of peak neonatal serum bilirubin. In addition, the presence of an abnormal neurologic examination at age 7 was studied among children with different peak bilirubin values. Finally, hyperbilirubinemic and non-hyperbilirubinemic children were compared for the presence of sensorineural hearing loss at age 8. The benefits of treating hyperbilirubinemia in otherwise healthy term infants are controversial. Since the clinical management of healthy term infants has changed little over the past decades, data of this age are still relevant. In fact, the absence of phototherapy as a treatment for hyperbilirubinemia makes these data even more valuable, as it would be very difficult to assemble a cohort of neonates with untreated hyperbilirubinemia today.

A second investigation, done in collaboration with personnel at the Epidemiology Branch of the National Eye Institute, is addressing the descriptive epidemiology of strabismus and congenital cataract. This condition is relatively common, affecting 2-3% of all children. Review of the literature reveals few studies describing risk factors for strabismus. The CPP conducted detailed neurologic examinations at ages 1 and 7, making it well-suited for the study of this condition.

A third investigation, done in collaboration with Dr. David Nagey of the University of Maryland, evaluated the predictive value of elevated maternal serum alpha fetoprotein for stillbirth in otherwise normal infants. In this study alpha fetoprotein levels in serum drawn at 14-20 weeks gestation from mothers delivering non-malformed stillborn infants were compared to levels in women delivering liveborn infants. Each stillborn case was matched to 4 liveborn controls for study site, race, gestation at which blood was obtained (± 3 days), and years elapsed since the serum was drawn (± 2 years). The fraction of cases and controls with alpha fetoprotein levels of greater than 3 multiples of the median was compared.

A fourth investigation studied the risk of childhood cancer following perinatal vitamin K exposure. Two previous reports have indicated that children who received this drug during the neonatal period are at approximately twofold increased risk of developing cancer. Since nearly every child born in the United States during the past several decades has received intramuscular vitamin K, up to half of all childhood cancer might be attributable to the drug. The CPP is an ideal cohort in which to study this issue, as it spanned the era when vitamin K exposure changed from being rare to being common.

Major Findings: Peak serum bilirubin was not associated with 7-year IQ in either black or white infants. Among white infants the IQ of children whose bilirubin value was ≥ 20 mg/dl was 105.0, compared to 103.4 for children whose bilirubin value was < 20 mg/dl. The corresponding values for black children were 91.0 and 93.3. Definitely abnormal neurologic examinations at age 7 were not more common among children whose peak neonatal bilirubin value was ≥ 20 mg/dl (4.5%) compared to those who were < 20 mg/dl (3.8%). However, the frequency of abnormal or suspicious neurologic examinations increased in a stepwise manner from 14.9% in children with bilirubin values < 10 mg/dl to 22.4% in those with peak bilirubin values of ≥ 20 mg/dl ($p < 0.001$). This increase was accounted for by minor, non-specific motor abnormalities.

In the alpha-fetoprotein analysis, the odds ratio for fetal death in pregnancies with serum alpha-fetoprotein values > 3.0 multiples of the median was 1.98 ($p = 0.018$). However, the sensitivity of elevated alpha fetoprotein for prediction of fetal death was only 8.5%; the specificity was 95.7%. In a modern-day population with a probability of late fetal death of 1%, the predictive value of a positive test would be only 2%, which is not sufficiently high to be of clinical utility.

There were 48 children in the CPP who developed cancer after the first day of life, including 16 who developed leukemia. This resulted in a cumulative incidence of cancer and leukemia of 1.1 and 0.4 per 1000, respectively, by age 7-1/2. Neonatal exposure to vitamin K was found not to be associated with the development of cancer during childhood (odds ratio 0.84, 95% confidence interval 0.41 to 1.71), or of leukemia (odds ratio 0.47, 95% confidence interval 0.14 to 1.55).

The results of the strabismus analyses indicate that 3% of the cohort developed esotropia and 1.2% developed exotropia. Esotropia was approximately twice as common in white as in black children, but exotropia did not differ by race. Both types of strabismus were more common in low birth weight infants. Maternal cigarette smoking during pregnancy was associated with an increased risk of both types of strabismus; this finding was independent of the birthweight-reducing effect of smoking.

Significance to Biomedical Research and the Program of the Institute: Each of these analyses involves a problem of relevance to child health. Treatment of jaundice results in large health care expenditure for the United States, due to the frequent extension of hospital stays for otherwise normal newborns. Furthermore, the need for this treatment is controversial. If it were to be shown that physiologic hyperbilirubinemia is not harmful to normal term infants, management of these infants might require reevaluation. Study of risk factors for strabismus will further our understanding of this common condition. The study of serum alpha fetoprotein will demonstrate the stability of this compound in long-term storage. It will also determine whether or not this test is a clinically useful marker for predicting stillbirth. The data on vitamin K provide important reassurance regarding the safety of this commonly used drug.

Preliminary results of the cataract analysis revealed few risk factors for the condition - other than low birth weight. Ongoing analysis will evaluate the association between cataract and other congenital anomalies.

Proposed Course of Project: Data analyses of the cataract and alpha-fetoprotein results is continuing, and results will be submitted to peer-reviewed journals.

Publications:

Chew E, Remaley N, Tamboli A, Zhao J, Podgor M, Klebanoff M. Risk factors for esotropia and exotropia. Arch Ophth 1994;112:1349-55.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00384-04 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Field Trial of Oral Cholera Vaccines in Bangladesh

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.D. Clemens	Branch Chief	EB, DESPR, NICHD
Other: M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS (if any)

Johns Hopkins School of Public Health (D.Sack); International Center for Diarrheal Disease Research, Bangladesh

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.6

PROFESSIONAL:

0.6

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project entails continuing analyses of data derived from a large-scale, placebo-controlled field trial of B subunit-killed whole cell (BS-WC) and killed whole cell-only (WC) oral vaccines against cholera, conducted between 1985-90 in the Matlab field studies area of the International Center for Diarrheal Disease Research, Bangladesh. The trial enrolled ca. 89,000 subjects for study. Analyses of the first three years of follow-up revealed that each vaccine conferred ca. 50% protection against cholera episodes detected among patients seeking care at medical facilities. During the past year, a paper on the fourth and fifth years of surveillance, during which no further protection was evident, was prepared. In addition, an analysis of serum immune responses to V. cholerae 01 among vaccine failures revealed that such vaccinees manifested poor responses, suggesting that impaired immunity may play a role in instances of vaccine failure.

During the past year, a large-scale case-control study was conducted to determine whether infection by Helicobacter pylori, a known cause of persistent hypochlorhydria, is a risk factor for cholera in Bangladesh. Preliminary analyses indicate that the risk of clinically severe cholera is elevated in H. pylori-infected individuals, but only when such individuals lack antecedent vibriocidal immunity to cholera. Infection by H. pylori was not a determinant of vaccine failure in the field trial. Analyses of H. pylori in children indicate that both household crowding and Hindu religion were associated with an elevation of risk, but that poorer nutritional status was not a risk factor for or a consequence of H. pylori infection.

Project Description:

Personnel: Dr. Clemens is the Principal Investigator of this project, with responsibility for the epidemiological design and execution of the study. Drs. Ahmed and Sack are epidemiologists who have collaborated on epidemiological aspects of the project. Mr. Rao has been responsible for computerized selection of cases and controls, and statistical analyses of the data.

Objectives: Specific goals of analyses conducted during the past year included: 1) evaluation of the efficacy of the vaccines during the fourth and fifth years of follow-up; 2) assessment of the relationship between serum immune responses to infection and the likelihood of vaccine failure; and 3) evaluation of the relationship between infection by Helicobacter pylori and the risk of cholera.

Methods Employed: This project entails continuing analyses of data derived from a large-scale, placebo-controlled field trial of B subunit-killed whole cell (BS-WC) and killed whole cell-only (WC) oral vaccines against cholera, conducted between 1985-90 in the Matlab field studies area of the International Center for Diarrheal Disease Research, Bangladesh. The trial enrolled ca. 89,000 subjects for study. After acquisition of informed consent and enrollment into the study, participants were randomized to receive 3 doses of BS-WC, WC, or an E. coli K12 strain placebo. Both active (community based) and passive (treatment center-based) surveillance techniques were employed to detect cholera infections, which were microbiologically confirmed via fecal microbiology. Subjects were also placed under demographic surveillance, using regular visits to homes on a weekly or biweekly basis. Sera were drawn from cholera cases at baseline and three weeks later, as well as from community controls.

To assess the relationship between H. pylori infection and the risk of cholera, cases with cholera were contrasted with controls without cholera for the presence of serum IgG anti-H. pylori antibodies, measured by ELISA. Both cases and controls were assigned to placebo in the oral cholera vaccine field trial.

Major Findings: Analyses during the past year demonstrated that serum vibriocidal responses to cholera infections in vaccinees ("vaccine failures") were lower than those in placebo recipients during the first year of follow-up. These data suggest that immunological hyporesponsiveness, even to natural cholera infections, may have accounted for a portion of vaccine failures and imply that such hyporesponsiveness may set an upper limit on vaccine efficacy attainable by any cholera vaccine. Additional findings included: 1) The vaccines conferred negligible protection during the fourth and fifth year of follow-up; 2) infection by H. pylori was associated with the risk of clinically severe cholera, but only in subjects lacking natural serum vibriocidal immunity; 3) H. pylori infection did not modify vaccine efficacy; and 4) crowding and Hindu religion were associated with a higher risk of H. pylori infection.

Significance to Biomedical Research and the Program of the Institute: With the spread of the seventh pandemic of cholera to South America in 1991, as well as recognition of the increasing mortality attributable to cholera in Africa and Asia, the importance of developing a vaccine effective against cholera has recently been underscored. The findings of this trial have provided the first demonstration that oral, as opposed to parenteral, vaccination is capable of providing long-term protection against cholera, validating human and animal data indicating that intestinal immunity is most relevant for protection against cholera and defining the direction of developmental efforts to produce new vaccines.

Proposed Course of Project: The coming year will be devoted to preparing manuscripts on H. pylori epidemiological studies.

Publications:

Clemens JD, Albert MJ, Rao M, Qadri F, Huda S, Kay B, van Loon FPL, Sack D, Pradhan BA, Sack RB. Impact of infection by Helicobacter pylori on the risk and severity of endemic cholera. J Infect Dis 1995;171:1653-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00385-04 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Epidemiology of Rotavirus Infections in Bangladesh

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS (if any)

James Gamble Institute for Medical Research, Cincinnati, OH (R.Ward)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project tests the hypothesis that humoral immunity to rotavirus infection, assessed in serum, is associated with the risk of rotavirus diarrhea in children aged under 2 years who reside in rural Bangladesh. Cases of rotavirus diarrhea were assembled from population-based, comprehensive surveillance of treated episodes of pediatric diarrhea in Matlab, Bangladesh during 1985-86. All rotavirus diarrhea episodes were serotyped with monoclonal antibodies after cultivating rotavirus isolates. Controls were similarly aged children randomly selected during four surveys of the Matlab community undertaken during 1985-86. Serological correlates of natural immunity to rotavirus diarrhea were assessed by contrasting acute-phase sera collected from the cases and sera from controls. Case-control comparisons revealed that: 1) serum IgG antirotavirus antibodies were correlated inversely with the occurrence of rotavirus diarrhea, suggesting a protective relationship; but 2) protective associations were not serotype-specific. As a second goal, we sought to determine whether breast feeding is associated with a reduced risk of rotavirus diarrhea in children under the age of 2 years. Comparisons of cases and community controls for antecedent histories of breast feeding revealed that: 1) breast feeding was associated overall with a reduced risk of rotavirus diarrhea, due to the protection conferred by exclusive but not partial breast feeding; but 2) after infancy, the direction of the relationship became reversed, with a higher risk of rotavirus diarrhea among breast-fed than non-breast-fed children.

Project Description:

Personnel: Dr. Clemens is the Principal Investigator of this project, with responsibility for the epidemiological design and execution of the study. Dr. Ahmed is an epidemiologist who has collaborated on epidemiological aspects of the project. Dr. Ward has conducted laboratory analyses of rotavirus isolates and sera. Mr. Rao has been responsible for computerized selection of cases and controls, and statistical analyses of the data.

Objectives:

- 1) To evaluate whether titers of serum IgG antirotavirus antibodies are associated with the risk of rotavirus diarrhea in children aged under 2 years who reside in rural Bangladesh, and to test whether such associations are serotype-specific.
- 2) To evaluate whether breast feeding is associated with a reduced risk of rotavirus diarrhea in children under the age of 2 years.

Methods Employed: These analyses were designed as case-control studies. Cases of rotavirus diarrhea were assembled from population-based, comprehensive surveillance of treated episodes of pediatric diarrhea in Matlab, Bangladesh during 1985-86. All rotavirus diarrhea episodes were serotyped with monoclonal antibodies after cultivating rotavirus isolates. Controls were similarly aged children randomly selected during four surveys of the Matlab community undertaken during 1985-86. Serological correlates of natural immunity to rotavirus diarrhea were assessed by contrasting acute-phase sera collected from the cases and sera from controls. IgG antirotavirus antibodies were tested with ELISA, and serotype-specific antibodies were measured with plaque-neutralization assays. The assessment of the relationship between breast feeding and the occurrence of rotavirus diarrhea was based on breast feeding histories obtained by interviews of parents or guardians.

Major Findings: The serological comparisons revealed the following findings: 1) serum IgG antirotavirus antibodies were correlated inversely with the occurrence of rotavirus diarrhea, suggesting a protective relationship; and 2) protective associations were not serotype-specific, but were both homologous and heterologous in nature. Comparisons of cases and controls for antecedent histories of breast feeding revealed that: 1) breast feeding was associated, overall, with a reduced risk of rotavirus diarrhea, due to the protection conferred by exclusive but not partial breast feeding; but 2) after infancy, the direction of the relationship became reversed, with a higher risk of rotavirus diarrhea among breast-fed than non-breast-fed children.

Significance to Biomedical Research and the Program of the Institute: Rotavirus infections are the major cause of dehydrating diarrheal illnesses in infants and young children worldwide. The development of vaccines against these infections require an understanding of the basis of natural immune protection against rotavirus diarrhea. Serological analyses from this study reaffirm the importance of natural immunity in determining the epidemiological pattern of rotavirus

diarrhea, but question whether serotype-specific immunity is of central importance either to natural protection against rotavirus diarrhea or to vaccine-induced protection. Among non-vaccine strategies for the control of rotavirus diarrhea, breast feeding has been proposed as a potential intervention. Our data indicate, however, that although a biological effect of exclusive breast feeding was evident among infants, the reversal of a protective relationship between breast feeding and the risk of rotavirus diarrhea during the second year of life yielded no net protection among the total group of children in the study.

Proposed Course of Project: This project has terminated.

Publications: Previously listed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00386-04 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Evaluation of a Water-Sanitation Intervention in Egypt

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS (if any)

UNICEF, Egypt

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The purpose of this field project is to evaluate the impact of a combined water-sanitation intervention on the occurrence of pediatric diarrhea and other targeted outcomes in a study population residing in the Assiut governorate of Upper Egypt.

20 villages, which together included a population of approximately 10,000 persons, were randomly assigned to receive the intervention (N=10) or no intervention (N=10). The intervention consisted of provision of India Mark II tubewells and household latrines, together with an educational package stressing personal hygiene and water behaviors. To assess the impact of the intervention, all families in these villages were followed with longitudinal surveillance during a one-year follow-up period.

Although the baseline incidence of diarrheal episodes (per 1000 person-days of follow-up) in children under three years of age was similar in intervention (29) and control (29) communities, after zero-time rates of diarrhea decreased 17% ($p < .0001$) in the intervention relative to the control communities. Protective associations were slightly higher for bloody diarrhea (25%, $p < .05$) and persistent diarrhea (26%, $p < .0001$), suggesting that the intervention might have reduced not only the risk of diarrhea but also the severity of diarrhea. In contrast, no impact on the occurrence of respiratory infections was evident, indicating that the decline of diarrhea in the intervention communities was not likely to be due to ascertainment bias. It was of particular interest that intervention communities exhibited a marked increase of per capita water consumption, but that the new pumps continued to produce water that was contaminated by fecal coliforms. There data suggest that the salutary effect of the intervention may have been mediated, in part, by increased water consumption, though the water itself was fecally contaminated. Analyses are now continuing.

Project Description:

Personnel: Dr. John Clemens of the Epidemiology Branch, DESPR, directs this study. Subjects were collected under contract by UNICEF to SPAAC, a private research firm in Cairo. Mr. Malla Rao of the DESPR is conducting the analysis.

Objectives:

1. To assess whether an intervention intended to provide tubewells and family latrines, as well as water-hygiene education, can improve salutary water-sanitation behaviors, as well as other measures of sanitation, such as environmental cleanliness, microbiological purity of stored water, and overall water usage.
2. To assess whether the intervention also results in a diminution in the rate of pediatric diarrhea in targeted villages.

Methods Employed: After assembly of consenting families and acquisition of informed consent, participating villages were randomly allocated, using stratified randomization, to intervention and control groups. Following implementation of the intervention, all participating families were visited on a weekly basis, and, at varying intervals, measurements of target outcomes were made with use of direct questionnaires, observations of relevant behaviors in the home environments, and observations of water use at the pumps, microbiological sampling of water in the homes and at the pumps.

Major Findings: The major finding obtained during the fiscal year was that children in the intervention villages experienced a 17% reduction in the rate of diarrhea, an effect that applied to both watery and non-watery diarrhea, and to acute and persistent diarrhea. Importantly, no protection was observed against acute respiratory infections, as would be predicted on epidemiological grounds. Moreover, the prevention of diarrhea associated with the intervention occurred despite increased per capita consumption of fecally contaminated water in the intervention communities.

Significance to Biomedical Research and the Program of the Institute: Pediatric diarrhea constitutes a major source of morbidity and mortality worldwide. Efforts to control pediatric diarrhea have, in the past, focused largely on improved case management, largely through promotion of oral rehydration therapy. However, these efforts have done relatively little to control the problems of dysentery and persistent diarrhea, for which preventive measures may be necessary. This evaluation of a pragmatic intervention which is already being incorporated into public health strategies by UNICEF, demonstrates that a multipronged water-sanitation program may indeed be capable of complementing the salutary effects of the case-management strategy.

Proposed Course: During the coming fiscal year, final analyses of the impact upon both proximate (e.g., behavioral, environmental) and ultimate outcomes (pediatric diarrhea and nutritional status) will be conducted. The project will also provide a rich set of data to address several issues in diarrheal disease

epidemiology, such as the impact of breast feeding on the risk of diarrhea, and the interaction of this association with environmental cleanliness; and the relationship between water use and water cleanliness with risk of pediatric diarrhea.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 HD 00389-04 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies of the Epidemiology of Pediatric Shigellosis in Bangladesh

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	J.D. Clemens	Chief	EB, DESPR, NICHD
Other:	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS (if any)

International Center for Diarrheal Disease Research, Bangladesh; Johns Hopkins University (F.Ahmed)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

.20

PROFESSIONAL:

.20

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

The purpose of this field project is to evaluate the epidemiological patterns and determinants of pediatric shigellosis occurring among residents in a research field site in rural Bangladesh who have had a documented recent exposure to a neighborhood resident having Shigella dysentery. 1934 children under 5 years of age, from 240 neighborhoods having a resident who presented for treatment of documented Shigella dysentery within 1 week of the initiation of active neighborhood surveillance, were assembled. Each of these children was followed with intensive active surveillance for one month, with a systematic schedule to ascertain symptomatic histories related to diarrhea, and to collect fecal specimens for microbiological characterization. Analyses have demonstrated that the occurrence of Shigella diarrhea was related to age (being highest in the third year of life), nutritional status (being higher in stunted children), season (being lowest during the monsoon season in the summer months), and breast-feeding status (being lower among breast-fed children, even up to 36 months of age). The presence of a latrine was not related to the risk, due to the fact that the presence of an unsanitary hanging latrine elevated the risk, while the presence of a sanitary latrine slightly depressed the risk. The lower risk of shigellosis in breast-fed than in non-breast fed children was almost entirely attributable to the increase in risk of shiglosis which occurred during the initial 3 months following cessation of breast feeding. When host and environmental factors are considered conjointly as predictors of pediatric shigellosis, only nutritional stunting, age, and feeding mode dominate as risk factors. Vitamin A status was not related to the risk of shigellosis. Future analyses will focus on determinants of persistent diarrhea among Shigella-infected children.

Project Description:

Personnel: Dr. Faruque Ahmed formerly of the Epidemiology Branch, DESPR, and now of Johns Hopkins University directed the data collection for this study. Subjects were collected while all investigators were scientists employed by the ICDDR,B in Bangladesh. Dr. John Clemens and Mr. Malla Rao of the DESPR are coinvestigators.

Objectives:

1. To describe the occurrence of Shigella diarrhea among Bangladeshi children residing in neighborhoods known to have been exposed to Shigella.
2. To ascertain both host and environmental determinants of Shigella diarrhea in these neighborhoods.
3. To determine the frequency and determinants of Shigella diarrhea progressing to persistent diarrhea in these neighborhoods.

Methods Employed: The project entailed assembly of 1934 children under 5 years of age, from 1335 families residing in 240 neighborhoods having a resident who presented for treatment of documented Shigella dysentery within 1 week of the initiation of active neighborhood surveillance. Following assembly of these children, each was followed with intensive active surveillance for one month. This surveillance included, at baseline, sociodemographic characterization, ascertainment of dietary and anthropometric status, and measurement of serum vitamin A. After baseline, subjects were visited according to a systematic schedule to ascertain symptomatic histories related to diarrhea, and to collect fecal specimens for microbiological characterization.

Major Findings: Analyses of the data thus far have demonstrated that the occurrence of Shigella diarrhea was related to age (being highest in the third year of life), nutritional status (being higher in stunted children), season (being lowest during the monsoon season in the summer months), and breast-feeding status (being lower among breast-fed children, even up to 36 months of age). The presence of a latrine was not related to the risk, due to the fact that the presence of an unsanitary hanging latrine elevated the risk, while the presence of a sanitary slightly depressed the risk. Interestingly, the protective effect of breast feeding was almost entirely attributable to the increase in risk of shigellosis which occurred during the initial 3 months following cessation of breast feeding. Clinically, almost half of detected episodes were non-bloody in character, emphasizing the difficulties in relying on dysentery as a marker of Shigella infections. Isolates that were multiply antibiotic-resistant were associated with more severe infections. Vitamin A-status was not associated with disease risk or severity.

Significance to Biomedical Research and the Program of the Institute: Pediatric diarrhea constitutes a major source of morbidity and mortality worldwide. Efforts to control pediatric diarrhea have, in the past, focused largely on improved case management, largely through promotion of oral rehydration therapy.

However, these efforts have done relatively little to control the problems of dysentery and persistent diarrhea, for which Shigella infections are etiologically of great importance and for which preventive measures may be necessary. Rational development of effective preventive interventions against Shigella requires a detailed appreciation of the epidemiology of this infection. This study therefore will provide information helpful both in the design of future interventions and in the targeting of high-risk populations for receipt of interventions.

Proposed Course: During the coming fiscal year, efforts will be devoted to completing manuscripts on determinants of disease severity and on Vitamin A as a determinant of disease risk and severity.

Publications:

Ahmed F, Clemens J, Rao M, Banik A. Family latrines and pediatric shigellosis in rural Bangladesh: benefit or risk? Int J Epidemiol 1994;23:856-62.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00392-04 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Fatal Injuries to U.S. Infants, 1983-1987

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	R.A. Brenner	Staff Fellow	EB, DESPR, NICHD
Other:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD
	H.W. Berendes	Director	DESPR, NICHD
	A.C. Trumble	Computer Specialist	CSB, DESPR, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Mndrs
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the spece provided.)

The National Center for Health Statistics linked birth and death records for U.S. infants provides the first opportunity to assess the demographic, socioeconomic and prenatal risk factors associated with deaths due to injuries in infants less than one year of age. U.S. studies of childhood deaths have been limited to the extremely sparse information available on standard death certificates. Both descriptive and risk factor analysis of infant deaths for 1983-1988 are being prepared for publication.

Of particular interest is a recent methodological analysis of infant injury deaths that are classified as "undetermined intent." By evaluating risk factor profiles of such injuries, it may be possible to classify these injuries as likely intentional or likely unintentional. This study has enormous public health implications, as such injuries could increase rates of child abuse in the less than one year age group by 10-15% in the U.S.

Project Description

Objectives:

- 1) To examine national fatal injury patterns among infants according to previously unknown risk factors, such as maternal education.
- 2) To determine the degree of bias introduced by potential misclassification of injuries of undetermined intent.

Methods Employed: Data for this study was provided from the National Center for Health Statistics linked birth/infant death data sets for the years 1983 through 1988. National, cause specific, injury death rates among infants less than one year of age were calculated. Univariate and multivariate analysis examined the effect of several maternal sociodemographic variables (e.g. maternal education) and perinatal variables (e.g. birth weight) on injury outcome.

Major Findings: The linked file data for the 1983-1988 national birth cohorts were pooled and analyzed. There were 6,674 deaths due to injury over this six year period, resulting in a death rate of 30/100,000 live births. Twenty one percent of deaths were classified as homicides, 75% as unintentional and 4% as undetermined intent. Potential risk factors ascertained from birth certificate information included both maternal and infant factors. Univariate analysis showed a significant association between each variable examined and injury death. Many of the characteristics were highly correlated, such as maternal age and marital status.

For most of the maternal factors, relative risks for deaths classified as undetermined intent were the same or higher than the RR's for deaths classified as intentional. For example, the RR of death for infants born to mothers with less than twelve years of education as compared to infants born to mothers with at least a college education was 23.3 for deaths of undetermined intent, 7.8 for intentional deaths and 6.0 for unintentional deaths. Adding the deaths with undetermined intent to intentional deaths increased the RR in this category to 9.0 from 7.8. Although this represents a relatively small change in relative risk, the absolute number of deaths now classified as intentional in the <12 years of education category would increase from 456 to 570, a 22% increase.

Another high risk category, with relatively small numbers of total births making it a practical target for interventions, is maternal age <15 years (n=61,053 from 1983-1988 out of a total of 22.6 million live births). The RR for death at ages <15 years as compared to ≥25 years was 8.6 for intentional injuries, 8.7 for injuries of undetermined intent and 3.7 for unintentional injuries. The RR resulting from combining deaths of undetermined intent with intentional deaths does not change enough to alter the predicted number of lives saved (difference of three deaths). However, deaths of undetermined intent are apparently both statistically and logically combined with deaths due to intentional injuries in these two examples.

Significance to Biomedical Research and the Program of the Institute: Injuries are the third leading cause of postneonatal death yet relatively little is known about the risk factors for injury death in this age group. The linked birth/infant death data set gives us a unique opportunity to examine risk factors

for injury death in this age group. These results, which identify several maternal factors which put infants at particularly high risk of injury death should help public health workers in targeting preventive interventions towards the families of those infants who are likely to be at greatest risk. In addition, preliminary results from the methodological analyses of injuries of undetermined intent indicate that these injuries are more likely intentional than unintentional in nature. These findings suggest that fatality rates from intentional injuries could be 10-15% greater than previously recognized.

Proposed Course: Initial descriptive findings were reported at the joint Society for Pediatric Research/American Pediatric Society/Ambulatory Pediatric Association meetings in 1993. Findings of the methodologic study on the classification of injuries of undetermined intent will be presented at the meeting of the American Public Health Association in October 1995. Several manuscripts, both descriptive and analytic, are currently being prepared.

Publications: None at this time.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00393-04 EB

PERIOD COVERED

October 1, 1994 through May 31, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Trends in Death Rates from Drowning among Children, 1971-1988

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R.A. Brenner

Staff Fellow

EB, DESPR, NICHD

Other: M.D. Overpeck

Epidemiologist

EB, DESPR, NICHD

COOPERATING UNITS (if any)

Johns Hopkins University (G.Smith)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.3

PROFESSIONAL:

0.3

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects

☐ (b) Human tissues

☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been completed.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 HD 00801-20 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies Based on the Medical Birth Registries of Norway and Sweden

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	A.A. Herman	Visiting Scientist	EB, DESPR, NICHD
Others:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
	J.F. Troendle	Staff Fellow	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

NIDCD (H.Hoffman); Univ. of Bergen, Norway (P.Bergsjø, L. Irgens); Univ. of Trondheim and Natl.Inst.of Publ.Hlth., Oslo, Norway (L.Bakketeig, A.Arntzen)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These studies have focused on: 1) size at birth as measured by head circumference, crown-heel length, and birth weight in relation to gestational age and perinatal mortality, 2) the tendency to repeat similar birth weight and gestational age in subsequent pregnancy outcomes to the same mothers, 3) perinatal mortality in relation to order of birth and size of sibship, 4) epidemiologic risk factors for preterm birth, 5) epidemiologic risk factors for small-for-gestational age births, 6) contribution of multiple births to perinatal mortality rates.

Project Description:

Objectives: These studies have been based on unique perinatal data sets, the Medical Birth Registries of Norway and Sweden. The objective is to compare United States data with that of other national population-based data sets from Scandinavia. These comparisons include birth weight-for-gestational age percentiles, birth weight-specific perinatal mortality rates and a variety of other sociodemographic, lifestyle, and health care comparisons.

Methods Employed: Analytic methods include indirect standardization of mortality rates by birth weight and gestational age, life-table analyses, discriminant analysis, examination of bivariate distributions using contour diagrams, fuzzy clustering of growth retarded and non growth retarded infants, non-parametric logistic regression, and other procedures applicable to population-based data. The epidemiologic approach is that of a "prospective" cohort design, which permits analyses by case-control, cross-sectional or longitudinal means.

Major Findings:

At low birth weight the variance of last menstrual period based gestational age is wide and the distribution is positively skewed toward higher values. In one study the variance of gestational age decreases rapidly as birth weight increases, skewness decreases and kurtosis increases in approaching the mean of the birth weight distribution. Some of the wider variance and positive skewness of gestational age at low birth weight appears to reflect heterogeneity of intrauterine growth, in which infants with high values of gestational age are growth retarded. We show by partitioning each birth weight group into two groups of infants with different gestational age distributions, that at low birth weight, infants with low gestational ages have higher neonatal mortality rates but lower fetal mortality rates than infants with a higher gestational age for birth weight. The differences in mortality described between small infants at different gestational ages suggest that infants with a high LMP-based gestational age have experienced a slower rate of intrauterine growth. Some authors interpret the distributional characteristics as indications of systematic error in last menstrual period based assessment of gestational age. It appears from this study that the extent of systematic error in the estimation of LMP based gestational age may have been overstated in the past. The fuzzy clustering approach used to classify infants into IUGR groups allowed us to bypass the categorical approaches of IUGR classification which are prone to misclassification. Analyses of Scandinavian and United States data showed that among preterm infants African-American excess infant mortality increases from twice to sixteen times that of whites as birth weight and gestational age increases. Among growth retarded infants the excess mortality increases from twice to eight times as birth weight and gestational age decreases.

Significance to Biomedical Research and the Program of the Institute: The studies using this rich data source have provided information on several issues pertinent to programs of the Institute. The "repeater" studies have helped to elucidate some of the etiologic problems of prematurity and low birth weight.

Studies of fetal and infant mortality, especially comparisons with available United States data sets, will aid understanding of the cause-specific mortality categories in which the United States rates may be higher than Scandinavian rates. The studies of IUGR classification allow for more complete examination of mortality and morbidity risk associated with growth abnormalities. The analysis of birth weight and gestational age specific mortality points to deficits in the care of preterm and growth retarded African-American infants.

Proposed Course of Project: Numerous research publications have resulted from these collaborative efforts on the Swedish and Norwegian Medical Birth Registries. Further collaborations using data from both of these registries are continuing with Dr. Per Bergsjö, Professor, Department of Obstetrics and Gynecology, University of Bergen, Norway. Dr. Allen Herman continues the analysis of perinatal mortality risks in relation to various size at birth measurements using fuzzy clustering and nonparametric logistic regression techniques.

Publications: None at present.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00861-13 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Assessment of In-Utero Fetal Growth Patterns in Relation to Outcome at Birth

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A.A. Herman

Visiting Scientist

EB, DESPR, NICHD

Other: K.F. Yu

Mathematical Statistician

BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

NIDCD (H.Hoffman); U.Trondheim, Norway (G.Jacobsen, L.Bakketeig); U. Bergen, Norway (P.Bergsjø, T.Markestad); U.Uppsala, Sweden (G.Lindmark, G.Ahlstein);

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects

☒ (b) Human tissues

☐ (c) Neither

☒ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This is a prospective study of risk factors associated with intrauterine growth retardation. Recruited were pregnant women before 17 weeks gestation at the University of Alabama in Birmingham and University of Trondheim, Norway (in collaboration with the Universities of Bergen and Uppsala) for the period of January 1986 through June 1988. The children are followed for five years to monitor cognitive, physical and behavioral development.

Project Description:

Objectives: These studies will evaluate the predictive value of several obstetrical tests, including diagnostic ultrasound measurements, in assessing fetal growth in-utero and in relation to outcome at delivery. Follow-up examinations of infants will evaluate morbidity status and developmental progress, including several growth parameters, at one and five years of age.

Methods Employed: This is a population-based study conducted in Alabama and Scandinavia. The population in Alabama focuses on the population of Jefferson County, which includes Birmingham. The health care for these women is provided for by the county health department in affiliation with the University of Alabama Medical School. The Scandinavian portion of the study is being conducted in three university towns (Bergen and Trondheim, Norway and Uppsala, Sweden), in which the medical care of pregnant women in the surrounding counties is provided for through each of the University Hospitals.

The goal of the study is to identify risk factors which will distinguish mothers having repeated small-for-gestational age (SGA) births from those who have an SGA birth unexpected from prior pregnancy history. The study populations are therefore based on the selection of high-risk samples, consisting of para 1 and 2 mothers with one or more of the following high risk characteristics:

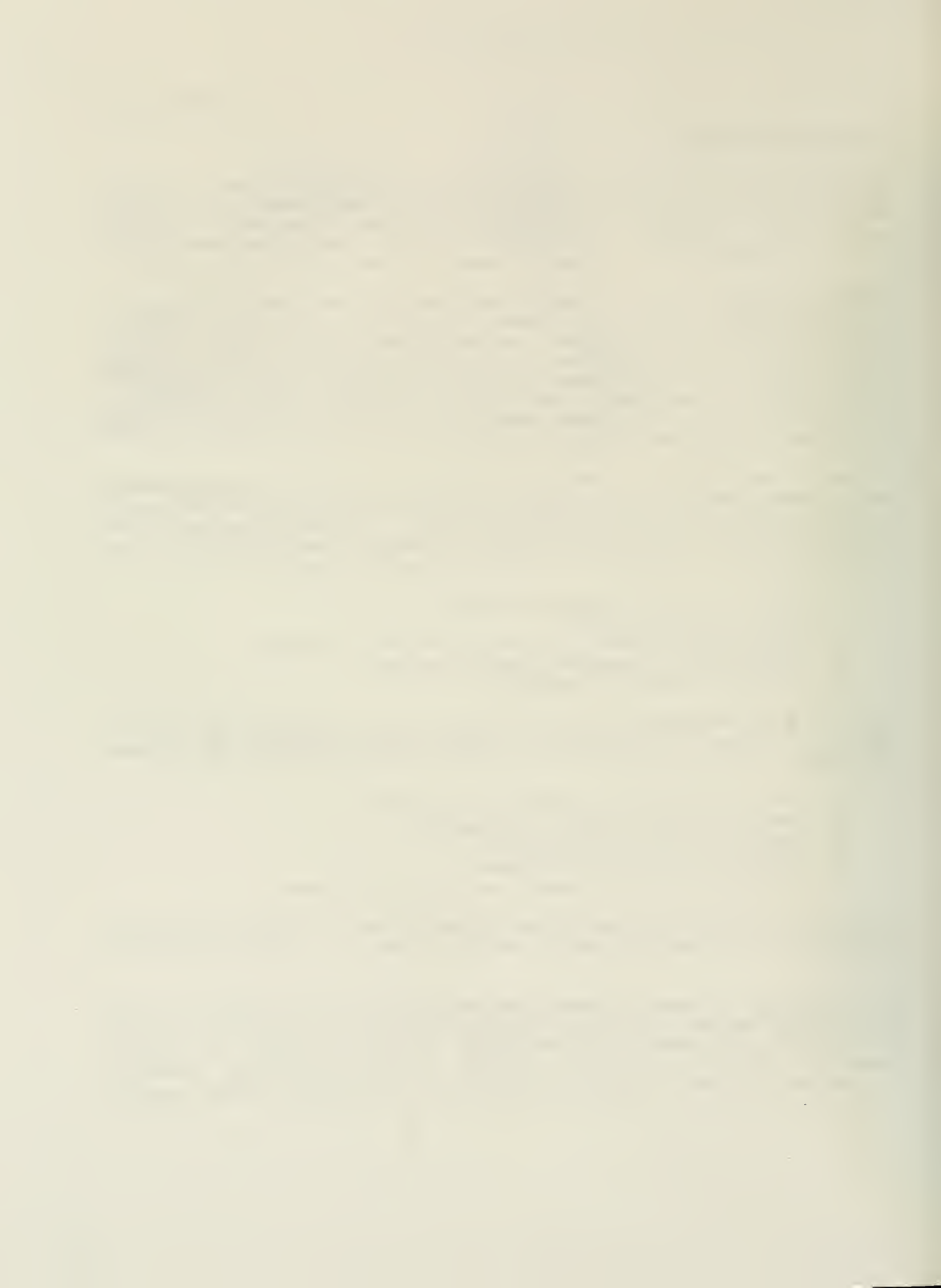
1. previous low birth weight delivery;
2. previous perinatal death;
3. serious renal disease or hypertension during pregnancy;
4. low maternal pre-pregnancy weight (<50 kg);
5. cigarette smoking at conception.

In addition to the above-listed risk factors which are common to the protocols used at all sites, the University of Alabama protocol includes the following additional criteria as well:

6. previous spontaneous abortions (2 or more);
7. previous preterm delivery (<37 weeks);
8. low maternal height (<157 cm);
9. alcohol drinking during pregnancy;
10. late onset of first prenatal care visit (26-32 weeks).

Several statistical techniques are being used to test for significant factors affecting fetal growth, including multiple linear and logistic regression analyses.

Major Findings: A number of manuscripts have been written, submitted and some published. They deal with the relationship between a psychological profile, maternal size and smoking and predicting intrauterine growth retardation, comparison of fetal biparietal diameter, head circumference, abdominal circumference and femur length by race and sex, the relationship between maternal



blood pressure, fetal growth retardation and preterm delivery; the study of maternal psychological characteristics and intrauterine growth retardation, as well as others. A special issue of Acta Obstet Gynecol will be published in 1995 summarizing findings from the first cycle of the study. The analyses of the five year follow up data has been initiated. The most important predictor of mental retardation at five years are the social circumstances of the family. Reduction in mean IQ is also predicted by size at birth and intrauterine growth retardation. Abstracts dealing with cognitive development at 5 years were presented at the 1995 Society for Gynecologic Investigation meeting and the 1995 Society for Epidemiologic Research meetings.

Significance to Biomedical Research and the Program of the Institute: Factors which affect intrauterine growth and which may provide insight into the pathophysiology of intrauterine growth retardation are of considerable significance to the mission of this Institute. Intrauterine growth retardation is a significant public health problem and associated with higher rates of perinatal infant mortality and increased morbidity.

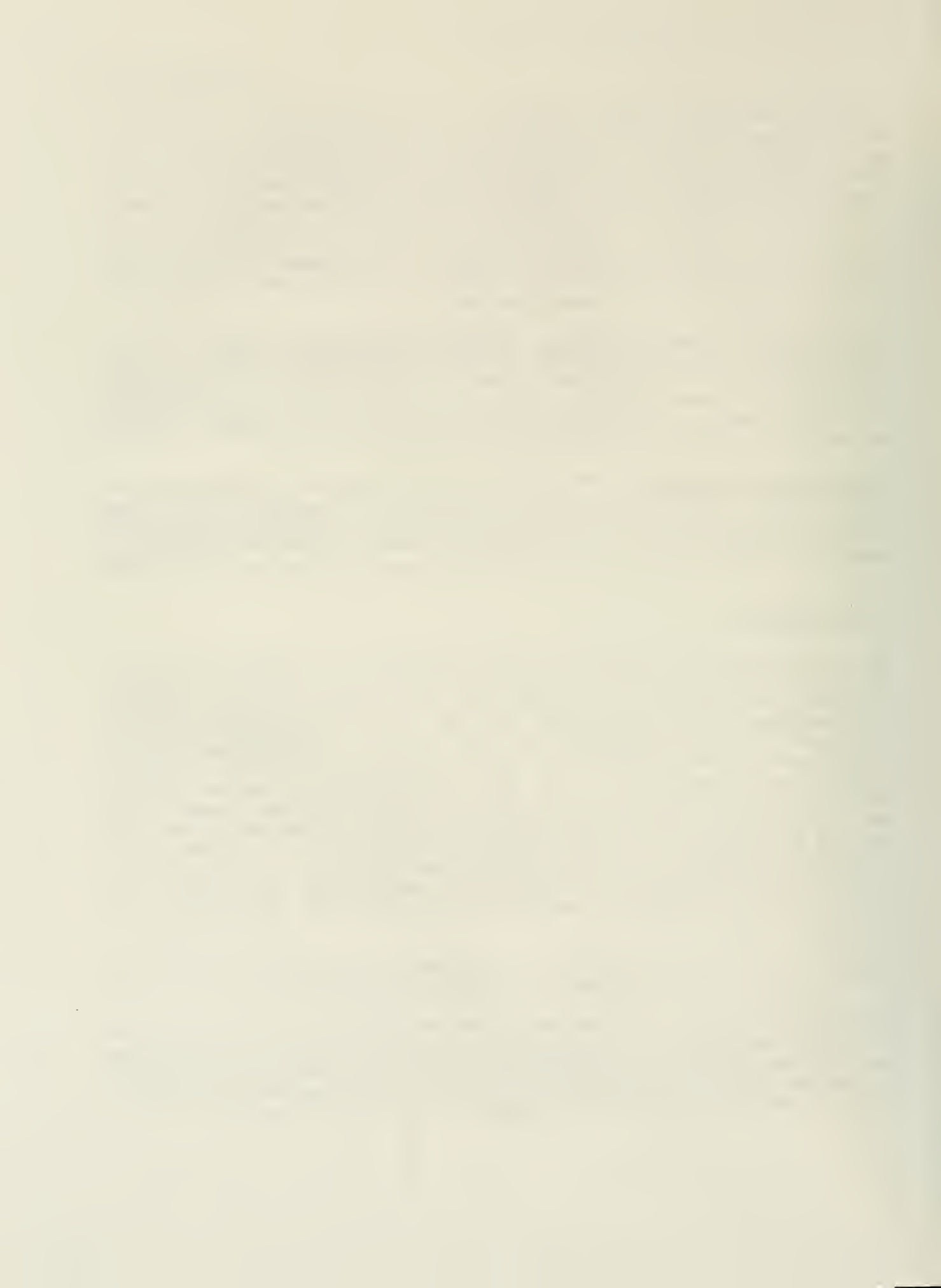
Proposed Course of Project: The children are being assessed as they become five years of age. Further analysis of this data are in progress. Dr. Herman together with scientists from both contracts will examine the intrauterine dynamics of fetal growth among infants with IUGR. In addition, the role of emerging risk factors such as psychosocial stress will be explored in these data sets

Contract Report

The data collection prospectively during pregnancy and follow-up of infants through the first year of life was supported by the University of Alabama in Birmingham under NICHD contract N01-HD-4-2811 and by the University of Trondheim in Norway under NICHD contract N01-HD-4-2803. These two contracts entitled "Successive Small-for-Gestational Age (SGA) Births: A Longitudinal Study of Fetal Growth and Perinatal Outcome" were initiated June 1, 1984. These two contracts terminated in FY 91. The data collection for the two new contracts entitled "Follow-up Study of Mental, Physical and Behavioral Development in Five Year Old Children" with the University of Alabama in Birmingham under NICHD contract N01-HD-1-3116 was initiated March 1, 1992 and with the University of Trondheim in Norway under NICHD contract N01-HD-1-3127 was initiated April 1, 1991. The FY 92 funding levels are \$276,632 and \$200,860, respectively. The current contract is devoted to the follow-up of children and their evaluation at five years of age.

The FY93 funding levels for NICHD contract N01-HD-1-3127 was \$220,643. Five year follow-up data collection efforts were completed during FY94.

The FY93 funding levels for NICHD contract N01-HD-1-3116 was \$291,272. Five year data collection efforts are expected to be complete by the end of FY94 but the contractor has asked for, and been granted an extension without additional costs to complete reporting requirements. The project is not expected to be funded in FY95. It was fully funded for the amount of the original contract.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 00872-10 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Factors Associated with Premature Births: Missouri Follow-Back Survey

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: A.A. Herman

Visiting Scientist

EB, DESPR, NICHD

Other: H.W. Berendes

Director

OD, DESPR, NICHD

COOPERATING UNITS (if any)

NIDCD (H.J.Hoffman); State Center for Health Statistics, Missouri Dept. of Health (G.Land, W.Schramm, J.Stockbauer, V.Pierson, B.Boley)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The objective is to obtain more accurate information relating to the very low birth weight (VLBW) infant, <1500 grams, than is now available from the United States vital records. This objective will be accomplished by the following: 1) mailing or administering a questionnaire to mothers of VLBW infants, mothers of fetal deaths, and a sample of mothers of moderately LBW infants (1500-2499 grams) and normal birth weight infants (\geq 2500 grams) in order to obtain and verify information from the prenatal, perinatal, and post-neonatal periods; 2) conducting telephone follow-up interviews on non-respondents and incomplete respondents, and a 10 percent sample of study mothers to obtain and/or verify information on the questionnaires; and 3) developing procedures for abstracting information from hospital and physician records, including otherwise unavailable or missing information on morbidity, lifestyle, and socioeconomic indicators of the study subjects. In addition, mortality and results of follow-up evaluations will be available through the first year of life for this birth cohort. Initial analyses of these data focused on the impact of very low birth weight and moderate low birth weight on cognitive development at one year, and the effect of maternal attitudes to weight gain on nutritional status and birth weight outcome.

Project Description:

Objectives: This study is intended to obtain more accurate information relating to the very low birth weight infant, <1500 grams, than is not available.

Methods Employed: This is a population-based study of all very low birth weight (VLBW) infants and fetal deaths occurring in Missouri during a 16 month time period (December 1, 1989 through March 31, 1991). Moderately low birth weight and normal birth weight controls have been matched to the VLBW infants. The Missouri Department of Health has gathered data by means of questionnaires from mothers of infants and fetal deaths, hospitals and physicians. These data were linked to birth - and infant death certificates. Surviving infants were followed up to one year of age. The revised Denver II screening examination was used to evaluate cognitive development at one year. In addition the use of well-baby health care was assessed and illnesses during the first year of life were ascertained from pediatric and maternal questionnaires.

Major Findings: Infant mortality rates among very low birth weight infants were very high. The surviving very low birth weight infants had an increased frequency of cognitive deficits at one year. Moderately low birth weight infants had higher rates of developmental delay at one year when compared to normal birth weight infants. Women who were embarrassed about being fat gained less gestational weight than those who were proud of being pregnant. Those who did not worry about size, and those who held the baby's health of paramount importance gained more weight during pregnancy.

Significance to Biomedical Research and the Program of the Institute: The study data will add considerably to our knowledge of risk factors and current morbidity conditions in the perinatal and post-perinatal periods. The initial results on cognitive development at one year has important implications for the impact of preterm birth and growth retardation on early schooling problems.

Proposed Course of Project: To continue the analyses of data and design a low term follow-up study of cognitive and physical health among preterm and growth retarded infants.

Publications: Two abstracts on risk factors for low birth weight were published at the 1994 American Public Health Association meeting.

Contract Report

The data collection has been supported by the Missouri Department of Health under NICHD contract N01-HD-6-2916. This contract entitled "Factors Associated with Premature Births: The Missouri Follow-back Survey" was initiated January 2, 1986. The FY 92 funding level has been \$200,000. The main objectives of this study are to provide new and current information about medical, sociodemographic, and behavioral/lifestyle risk factors of mothers who have VLBW infants and fetal deaths compared to those who have either moderately low birth weight or normal birth weight infants. The FY 94 funding for N01-HD-6-2916 was \$150,000 and concludes the funding for the data collection phase.



DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02500-03 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

ETEC Seroepidemiology Evaluations in Alexandria, Egypt

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: J.D. Clemens

Branch Chief

EB, DESPR, NICHD

Other: M.R. Rao

Visiting Scientist

EB, DESPR, NICHD

COOPERATING UNITS (if any)

NAMRU-3 Unit, Cairo, Egypt (R.Abu Elyzeed, B.Kay, L.Peruski); University of Alexandria (A.Mourad, M.Gaafar, M.Shasly); University of Goteborg (J.Holmgren, A-M.Svennerholm)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.6

PROFESSIONAL:

0.6

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Enterotoxigenic Escherichia coli (ETEC) diarrhea is hyperendemic among young Egyptian children, with an estimated cumulative incidence of 2.4 symptomatic ETEC episodes from birth to 36 months, and a balanced distribution of toxin phenotypes. These features make it logical to develop a field site for the evaluation of ETEC epidemiology and ETEC vaccines in Egypt. Recently, we have begun a collaborative project to develop a site in Abees (near Alexandria) for the study of pediatric diarrhea.

This project is following a pediatric cohort with the following aims: 1) to determine the age-specific incidence rate of ETEC diarrhea from birth to 35 months, by toxin and colonization factor (CFA) phenotype; 2) to ascertain the strength of attribution of diarrheal symptoms to fecal isolation of ETEC; 3) to evaluate the protective relationship between titers of serum IgG antibodies to ETEC toxins and CFAs and the risk of diarrhea due to ETEC manifesting these virulence factors. Work began in June, 1993 on protocol design, data form development, development of computer systems for data entry and management, and preparation of manuals of procedures. In November, 1993 enrollment of subjects began. To date, approximately 190 children have entered the study.



Project Description:

Personnel: Dr. John Clemens of the Epidemiology Branch, DESPR, is the principal investigator of this project, with responsibility for the epidemiological design, execution, and analysis of the study. Implementation of the study is additionally being undertaken by scientists at the NAMRU-3 Unit in Cairo (Drs. R.Abu Elyzeed, B.Kay, L.Peruski) and the Departments of Microbiology (Prof. A.Mourad) and Preventive Medicine (Prof. M. Shasly) of the University of Alexandria. Professors J. Holmgren and A-M. Svennerholm of the Department of Medical Microbiology of the University of Goteborg are consulting on microbiological aspects of the study. Mr. M.R. Rao of NICHD is responsible for the overall design of computerized data management.

Objectives:

1. To determine the age-specific incidence rate of ETEC diarrhea from birth to 35 months, by toxin and colonization factor (CFA) phenotype;
2. To ascertain the strength of attribution of diarrheal symptoms to fecal isolation of ETEC;
3. To evaluate the protective relationship between titers of serum IgG antibodies to ETEC toxins and CFAs and the risk of diarrhea due to ETEC manifesting these virulence factors.
4. To establish, as a basis for future field trials of ETEC vaccines, the field, laboratory, and data management capabilities for detecting and etiologically characterizing ETEC diarrhea by toxin and CFA phenotype and for evaluating serological responses to ETEC virulence factors.

Methods Employed: The study is assembling a cohort of newborns and children under 24 months of age, and, with twice-weekly visits to homes, ascertains diarrheal events, which will be characterized microbiologically for ETEC (including specific ETEC virulence factors) and other conventional enteropathogens using conventional techniques. Blood is collected by fingerstick every three months, and sera will be assayed for anti-ETEC IgG antibodies, for correlation with the ensuing incidence of diarrhea caused by ETEC exhibiting homologous virulence factors.

Major Findings: None to date (project is in progress).

Significance to Biomedical Research and the Program of the Institute: It has been estimated that among children under 5 years in the developing world nearly 70 million episodes of diarrheal disease due to ETEC occur each year, accounting for approximately 700,000 deaths. Although crude, these estimates highlight the high disease burden of ETEC infections in the developing world, and indicate that a vaccine which is effective against ETEC would be of great public health benefit to children in these settings. Accordingly, the development and testing of effective vaccines against ETEC diarrhea has been identified as a specific priority of the Programme for Vaccine Development of the World Health



Organization. Critical to the testing of future ETEC vaccines is the development of field sites in which ETEC is endemic, and in which the epidemiology of ETEC is characterized in suitable detail for estimation of sample size requirements and other aspects of study design. In this regard, it is important that the occurrence of ETEC by toxin and CFA phenotype be known, since vaccine protection is expected to be homologous with respect to these virulence factors, and since the distribution of these factors is known to vary substantially in different areas.

The present research will be conducted in Abees, Egypt, a rural, agricultural area (population base of approximately 20,000) located at the mouth of the Nile delta within 20 Km of Alexandria, Egypt's second largest city. This research will be conducted as a collaboration between the National Institute of Child Health and Human Development (NICHD) of the U.S. National Institutes of Health; the U.S. Navy, through the research unit in Cairo, Egypt (U.S. Navy Medical Research Unit No. 3: NAMRU-3); and the Faculty of Medicine, Alexandria University, Alexandria, Egypt (Departments of Microbiology and Community Medicine). The research will develop a community-based diarrheal disease study site that will ultimately serve as a site for evaluation of vaccines against ETEC and other pediatric enteric infections. In addition to providing important epidemiological information about childhood ETEC diarrhea, this study will augment the field and laboratory infrastructure of this field site, so that it will be suitable for the testing of future candidate vaccines against ETEC.

Proposed Course: This is envisioned as a four-year project.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02502-03 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

NICHD-Health Research Board of Ireland Neural Tube Defects Study

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: J.L. Mills Chief, Pediatric Epidemiology Section EB, DESPR, NICHD
Other: M.R. Conley Computer Specialist EB, DESPR, NICHD

COOPERATING UNITS (if any)

Health Research Board of Ireland and Trinity College, Ireland

LAB/BRANCH

Epidemiology Branch

SECTION

Pediatric Epidemiology Section

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.8

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☒ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The Epidemiology Branch (DESPR) is conducting a number of studies in collaboration with the Health Research Board and Trinity College, Ireland. These investigations are designed to determine the biochemical mechanisms by which folate reduces the risk for neural tube defects. Data and blood samples have been collected on a large proportion of Irish women delivering babies in Dublin. Samples from women whose pregnancy ended in the delivery of a child with a neural tube defect and control women whose pregnancy ended in the delivery of a normal child are being studied. Various aspects of folate metabolism and other nutritional measures are being examined. The identification of vitamin B12 as a significant factor in preventing neural tube defects led us to examine the metabolic pathways which involve both B12 and folic acid. We have demonstrated that at lower levels of B12, women carrying a fetus with an NTD have significantly higher levels of homocysteine than women carrying a normal fetus. Introductory genetic studies have been very promising. We have identified an abnormal gene for an enzyme important to homocysteine metabolism in some NTD subjects and their parents.

Project Description: Dr. James Mills of the Epidemiology Branch, DESPR directs the NICHD-Health Research Board of Ireland Neural Tube Defects study. Subjects for this study have been identified in the Dublin area by our collaborators in the Health Research Board under the direction of Dr. Peadar Kirke. A biochemical investigation is being done in the laboratory of Professor John Scott at Trinity College, Dublin. Ms. Mary Conley of the DESPR is responsible for data center and Dr. Jack Lee of the DESPR is the statistician.

Objectives: To identify the mechanism by which folate prevents neural tube defects, and to characterize the population at risk.

Methods Employed: Ongoing biochemical assays are looking for differences in mothers who have had NTD children both during affected pregnancies and at other times. Genetic studies to identify markers for abnormal enzyme function are beginning.

Major Findings: Having demonstrated abnormal homocysteine metabolism in mothers carrying neural tube defect offspring, we are pursuing the question of which of several enzyme pathways which could cause this abnormality is responsible. We have determined that a genetic abnormality in a relevant enzyme accounts for some (but not all) NTDs.

Significance to Biomedical Research and the Program of the Institute: We have taken an important step in identifying an initial genetic component which acts with environmental components (folic acid and vitamin B12 levels) to produce neural tube defects. If it becomes possible to identify women at risk using markers for abnormal enzyme function, it will be possible to target vitamin prophylaxis. This would be of significant clinical and economic benefit.

Proposed Course of Project: Current plans include further genetic studies in search of abnormalities explaining more NTD cases. We will continue to study mothers of NTD children during an affected pregnancy, a non-affected pregnancy, and in a non-pregnant state. We are developing cell culture techniques to study enzyme function directly. The folic acid dose and duration of therapy necessary to raise a sub-optimal red cell folate level to an optimal plateau is now being studied in the female employees of a Dublin hospital.

Publications: Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG, Scott JM. Homocysteine metabolism in pregnancies complicated by neural-tube defects. Lancet 1955;345:149-51.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 HD 02503-03 EB

PERIOD COVERED

October 1, 1994 through March 31, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

1991 Follow-up National Maternal and Infant Health Survey

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: M.D. Overpeck

Epidemiologist

EB, DESPR, NICHD

COOPERATING UNITS (if any)

National Center for Health Statistics (M.D. Kogan and L.A. Fingerhut)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects

☐ (b) Human tissues

☐ (c) Neither

☐ (a1) Minors

☒ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

This project has been completed.

Project Description

Objectives: To examine the cumulative risk of injury among children from birth to three years old, and to provide national-level cause-specific estimates of non-fatal injuries for this age group.

Methods Employed: Respondents to the maternal questionnaire of the 1991 NMIHS-LF were asked to describe all injuries that required a doctor's or nurse's care. Responses were categorized by external cause of injury. Data were analyzed using software for the analysis of complex multistage surveys (SUDAAN) to be representative of the U.S. national distribution for 1988 live births.

Major Findings: Almost 25% of children were reported to have ever been seen by a medical provider for an injury. Among children who had an injury, 24.5% had more than one. Boys were more than twice as likely as girls to have had an injury. Children who received their medical care from private physicians' offices or HMO's and those in upper socioeconomic levels were more likely to have had a medically-attended injury. Falls, burns, poisoning, and being struck or cut were the most frequently reported cause.

Significance to Biomedical Research and the Program of the Institute: Injuries are the primary cause of death for children in the United States. Data on non-fatal injuries are extremely limited and non-representative. Most studies are based on emergency room data without a known population at risk. This study provides new data with demographic risk factors in a representative population-based U.S. sample.

Proposed Course: Work completed.

Publications: Kogan MD, Overpeck MD, Fingerhut LA. Medically-attended non-fatal injuries among preschool children: National estimates. Am J Prev Med 1995;11:99-104.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02505-03 EB

PERIOD COVERED

October 1, 1994 through December 31, 1994

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Infectious Disease Mortality during Infancy: United States, 1987

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	J.S. Read	Senior Staff Fellow	EB, DESPR, NICHD
Other:	J. Troendle	Staff Fellow	BMSB, DESPR, NICHD
	M.A. Klebanoff	Research Medical Officer	EB, DESPR, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.05

PROFESSIONAL:

0.05

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

This project is terminated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02506-03 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Lack of Age-Appropriate Immunization among Infants Born in District of Columbia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R. Brenner	Staff Fellow	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief	EB, DESPR, NICHD
	B. Simons-Morton	Health Research Specialist	PRB, DESPR, NICHD

COOPERATING UNITS (if any)

Children's National Medical Center; D.C. Commission of Public Health; D.C. General Hospital; Georgetown University; Howard University; University of the District of Columbia

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.9

PROFESSIONAL:

0.9

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

As part of the "NIH/DC Initiative to Reduce Infant Mortality" a program of research aimed at increasing the proportion of infants and children in the District of Columbia who are age-appropriately immunized has been developed. This program of research includes both epidemiologic and intervention (efficacy) studies. The phase I studies include needs assessment and feasibility studies in addition to a study examining determinants (as measured at birth) of immunization status at 3 and 7 months of age. The findings from this phase will be used in the planning and execution of an intervention study in phase II.

Investigators include epidemiologists, behavioral scientists, health care providers and administrators. The program of research was developed with input from the variety of perspectives represented by the investigators and the proposed studies have the potential to benefit from a unique blend of epidemiology, behavioral science, and public health and health care practice. It is our intent to develop interventions and identify determinants that are relevant to public health practice in inner cities across the US.

This report focuses on the phase I epidemiologic studies.

Project Description:

Objectives: 1) Identify prospectively (at the time of birth) determinants of age-appropriate immunization among infants and children born to residents of the District of Columbia. 2) Assess the association between lack of age-appropriate immunization and other poor health outcomes.

Methods Employed: A birth cohort will be assembled from nursery log books. Baseline information will be gathered via face to face interviews with the mother during her hospital stay or, if this is not feasible, within two weeks of the delivery.

Demographic information as well as "consumer characteristics" (e.g. knowledge, attitudes, and beliefs concerning immunizations, well child care and childhood illness) will be collected at this time. Infants/Mothers will be contacted at 3 months and at 7 months to assess immunization status and other health outcomes. Medical records will be reviewed for all health care visits. Information regarding health care provider characteristics and health care system characteristics will be gathered for all identified sites of patient care. (Methods for determining the current residence of infants and for retrospectively ascertaining immunization status and other health outcomes will also be addressed in the phase I studies.)

To determine predictors of delayed initiation of immunization, infants who are appropriately immunized at three months of age will be contrasted to those who are not. Similarly, to determine predictors of attenuation of age-appropriate immunization status, infants who are initially immunized at 2 months of age but then fail to complete the primary series in a timely fashion will be contrasted with infants who are appropriately immunized at 7 months.

Major Findings: Data not yet available for analysis.

Significance to Biomedical Research and the Program of the Institute:

Several factors make this an extremely important project: 1) Assurance of age-appropriate immunization is a national public health objective. The National Health Promotion and Disease Prevention Objectives (Healthy People 2000) include a national goal of at least 90% completion of the recommended immunizations by 24 months of age. 2) Surveys conducted in the District of Columbia in 1990 and 1991 revealed that less than 40% of children had received all recommended immunizations by 2 years of age. 3) Immunization is only one of many important components of preventive pediatric health care for infants and children. Interventions to improve immunization status may have the added benefit of increasing utilization of preventive health care, linking infants and children to systems that may provide continuity of care. 4) Studies in other cities have identified delayed receipt of the initial two month vaccine as the strongest risk factor for lack of age appropriate immunization at two years. This study will be one of the first to prospectively examine factors related to delayed initiation of immunization. In addition, it is desirable to identify determinants of immunization status prospectively (at the time of delivery) such that high risk families can be identified for targeted interventions.

Proposed Course of Project: The hospital-based cohort study will be conducted over an eighteen month period. Study enrollment began in August of 1995 and will continue through the Spring 1996. Based on these recruitment projections, the 3 and 7 month interviews will be completed by the Fall of 1996 and medical record reviews will be completed early in 1997.

Publications: None yet.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02507-03 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Prostaglandin Excretion in Preeclampsia

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	J.L. Mills	Chief, PES	EB, DESPR, NICHD
Other:	R.J. Levine	Expert	EB, DESPR, NICHD
	J.D. Clemens	Chief, EB	EB, DESPR, NICHD

COOPERATING UNITS (if any)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

A disturbance of prostaglandin metabolism is currently believed to be one of the major mechanisms in the pathogenesis of preeclampsia. Most previous studies of this theory, however, have included only women who had already developed clinically overt disease. In order to investigate whether prostaglandin abnormalities precede the onset of preeclampsia, we have designed a nested case-control study using stored urine specimens prospectively collected as early as thirteen weeks gestation from women participating in the NICHD Trial of Calcium to Prevent Preeclampsia (CPEP). In this study, we will compare the urinary excretion of prostaglandin and thromboxane in preeclamptic women before the diagnosis of preeclampsia to that in normal pregnant women at comparable gestational ages. Because half of the women in the CPEP trial will be taking two grams of oral calcium daily, we will also be able to examine the effect of oral calcium supplementation on the excretion of these prostaglandin metabolites.



Project Description: Dr. James Mills, Dr. Richard Levine, and Dr. John Clemens are collaborating on this project. The laboratory analyses will be performed by Dr. Jackson Roberts at Vanderbilt University.

Objectives: To determine whether an imbalance of prostaglandin metabolism precedes the clinical onset of preeclampsia.

Methods Employed: Vanderbilt University is assaying specimens (blinded to status) from women who developed preeclampsia and normal control women. Thus far, nearly 200 urine specimens have been assayed for the prostacyclin metabolite 2,3-dinor-6-keto-PGF₁ α and the thromboxane metabolite 11-dehydro-TxB₂.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute: Preeclampsia occurs in 5-10% of all pregnancies, and causes substantial maternal and neonatal morbidity. Determining the pathogenic mechanisms underlying this disease will assist in development of rational prediction tests and therapies.

Proposed Course: Samples will continue to be analyzed until the CPEP trial is completed. Then the code will be broken and the final analysis will be performed.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02510-03 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Diet, Maternal Nutritional Status, Blood Pressure and Fetal Growth

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	N. Tafari	Visiting Scientist	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief	EB, DESPR, NICHD
	H.W. Berendes	Director	DESPR, NICHD
	Y. Johnson	IRTA Fellow	EB, DESPR, NICHD

COOPERATING UNITS (if any)

Ethiopian Nutrition Institute (Z.W. Gabriel, E. Woohib)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The study was sponsored by the City Council of Addis Ababa as part of health and nutritional assessment of low income families prior to planning a food supplementation program for women and children. This particular program was initiated because of sudden increase in the price of essential food items following implementation of government policies that preferred state control of production and distribution of all essential commodities.

The study of the effect of home diet on fetal growth arose from the observation that in Ethiopia, where severe deficiency in food energy is prevalent, there appears to be no corresponding deficit in fetal growth. This observation is at variance with the generally held view that dietary energy deficit during pregnancy leads to fetal growth retardation. However, several lines of recent evidence suggest that fetal growth is protected through adaptive processes. Among possible adaptive mechanisms area: 1) higher physical work efficiency; 2) greater mobilization of maternal energy reserves (fat deposits); and 3) hemodynamic adjustments to maintain adequate uterine perfusion.

Data analysis to date showed that the dietary energy deficits in low income families were associated with deficiencies in nutritional status of the gravida during the third trimester and the puerperium as reflected by reduced skinfold thickness and body mass index. Third trimester diastolic blood pressure was significantly reduced in poorly nourished gravida. Although a statistically significant difference of 180 g in birth weight was detected between the offspring of the undernourished and adequately nourished, it is not yet clear whether this difference is due to diet alone.

Project Description

Objectives: The objective of the project is to investigate the effect of home diet on fetal growth in a population with long term energy undernutrition.

Methods Employed: The study was part of a health and nutrition survey of low income families in Addis Ababa during 1987-88 when it was felt that food availability at the level of the household was reduced as a result of disturbance of the market forces by government policies that preferred state control of production and distribution of all essential commodities. The study was conducted in four community-based primary health care facilities serving the poor, and in one privately financed prenatal health service serving the affluent. Dietary energy, protein and selected nutrient intake were estimated from 4-consecutive-day weighed measurements of the home diet. Individual food items were weighed before cooking. The meal is weighed again before consumption. The daily household consumption was estimated from the difference in weight between food prepared and left over food. The nutrient content of the home diet was estimated using values of the "National Food Composition" tables. Household energy intake was expressed as multiple of basal metabolic rate (BMR). The "household basal metabolic rate" (HBMR) was the sum of the individual member's BMR and was estimated from published tables (WHO, Tech Report Series 724, 1985). According to WHO the *survival energy requirement* that allows only for the minimum of activities is 1.27 times BMR. Nutritional status of the pregnant woman and her family was assessed using selected anthropometric measurements, including weight, height and skinfold thickness at selected sites. Other variables considered include weight, skinfold thickness and blood pressure changes during pregnancy and puerperium.

Major Findings: Preliminary data analysis on women who participated in the home diet survey is completed. Women attending community-based prenatal clinics had lower socioeconomic profile than women using private prenatal facilities as reflected by lower income and low food energy intake. The poor gravida also tended to be younger with higher parity. The low energy intake in these women was reflected in their poor nutritional status -- low body mass index and reduced skinfold thickness. When the gravida were stratified into those with household energy intake to expenditure ratio <1.27 and ≥ 1.27 the women with lower energy intake to expenditure ratio had a significantly lower diastolic blood pressure and the mean birth weights of their newborns was 180 g lighter.

As anticipated, the deficit in birth weight is smaller than the actual deficit in food energy intake and the degree maternal undernutrition as assessed by anthropometric measurements. Data exploration and analysis on the entire cohort is in progress in an attempt to identify adaptive mechanisms that tend to minimize the effect of prenatal food energy deficiency on fetal growth.

Significance to Biomedical Research and the Program of the Institute: This cohort offers a unique opportunity to study the effect of maternal food energy deficit to near starvation level, the resulting nutritional status and blood pressure changes during the second half of pregnancy on fetal growth. If the study yields information on adaptive mechanisms protecting fetal growth, such knowledge may help health planners to target pregnant women who lack these

adaptive traits. Since the women were followed through delivery to the end of the puerperium it can also provide information on the additional effect of pregnancy on postnatal nutritional status of undernourished women.

Proposed Course of Project: Data analysis and manuscript preparation will continue during the next fiscal year.

Publications: None yet.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02511-02 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Immunogenicity of Routine Childhood Vaccines in HIV Positive Children

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	J.S. Read	Senior Staff Fellow	EB, DESPR, NICHD
Other:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD

COOPERATING UNITS (if any)

FDA (B.Anthony); NERI (D.Brambilla); NIAID (M.G.Fowler); Columbia University (J.Pitt); University of Illinois (K.Rich)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.25

PROFESSIONAL:

0.25

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The immunogenicity of routine childhood vaccines in HIV-infected children is not well understood and may be substantially less than in non-infected children. It is important to determine the immunogenicity of such vaccines in this population and thus identify the most immunogenic vaccines and vaccine schedules in this population. Data from the Women and Infants Transmission Study (WITS) will be analyzed.

Project Description:

Objectives: This study was designed to address the following hypotheses: 1) The magnitude and duration of the immune response of HIV-infected children to these vaccines will be less than that of noninfected children; 2) Among HIV-infected children, the serologic response to these vaccines will be inversely related to the severity of HIV-related symptoms, i.e., HIV-infected children who are clinically symptomatic at the time of vaccination will respond less well than asymptomatic HIV-infected children; and 3) Among HIV-infected children there may be clinically significant differences in the magnitude and duration of the immune responses to the vaccines.

Methods Employed: Children of HIV-infected women enrolled in the Women and Infants Transmission Study (WITS) (N=443 as of March 1, 1993) will be eligible for inclusion in this study. All children remain under surveillance until 36 months of age, during which time all routine childhood immunizations are administered and venipuncture is performed on a regular basis. Data regarding types of immunizations received, dates of immunizations, and vaccine manufacturers are stored in the WITS database.

Major Findings: Primary analyses will compare magnitude and duration of the immune responses of HIV-infected children to DTP and Hib vaccines to those of noninfected children. Further analyses will compare the serologic responses to these vaccines in symptomatic and asymptomatic HIV-infected children.

Significance to Biomedical Research and the Program of the Institute: HIV infection has become the most common cause of immunodeficiency in children in many parts of the world. In the U.S., the number of individuals in the pediatric age group infected with HIV is rapidly increasing. Vaccine-preventable diseases continue to cause significant morbidity and mortality in HIV-infected children in the U.S. Because HIV-infected children will remain at risk for vaccine-preventable diseases until these diseases are eradicated, it is important to determine the immunogenicity of routine childhood vaccines in this population and, eventually, to identify the most immunogenic vaccines and vaccine administration schedules in this population.

Proposed Course of Project: The serological testing is anticipated to be completed within the next fiscal year.

Publications: None yet.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02512-02 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Birth Certificate Linkage to Growth and Health Measures using NHANESIII

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M.D. Overpeck

Epidemiologist

EB, DESPR, NICHD

COOPERATING UNITS (if any)

National Center for Health Statistics (R.Kuczmarski, G.Gay, J.Findlay)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The third National Health and Nutrition Examination Survey (NHANESIII) has collected descriptive data, physical measures, and biomedical samples (blood and urine) on a representative U.S. sample of children at ages two months through six years with oversampling for blacks and Mexican Americans. Linkage of the extensive data from NHANESIII to the child's birth certificate to use the prenatal and birth information has begun. All of the states were sent requests for the relevant birth certificates with the signed permission of parents of the examined child. The data from the first three states have been received. These data are being used to test the computerized matching of NHANESIII and birth certificate data.

Results of the linkage will support research efforts to do the following: 1) To determine if low birth weight or premature children have subsequent poor health as measured by biomedical markers in a representative national sample of children through age six; 2) to assess the effects of ethnic/racial and socioeconomic factors on these outcomes; 3) to develop normative growth and other biomedical scales for a U.S. representative population, and black and Mexican-American children, according to a risk profile at birth; and 4) to develop comparisons between maternal and proxy reports of children's risk factors at birth and actual measurements for use in other studies.



Project Description:

Objectives: 1) To determine if low birthweight or premature children have subsequent poor health as measured by biomedical markers in a representative national sample of children through age six; 2) to assess the effects of ethnic/racial and socioeconomic factors on these outcomes; 3) to develop normative growth and other biomedical scales for a U.S. representative population, and black and Mexican-American children, according to a risk profile at birth; and 4) to develop comparisons between maternal and proxy reports of children's risk factors at birth and actual measurements for use in other studies. Major outcomes of interest will include 1) weight and height growth scales by risk profiles at birth; 2) effects of passive smoke exposure in the household controlling for prenatal factors; and 3) other health measures collected both by questionnaire and biomedical measurement.

Methods Employed: The third National Health and Nutrition Examination Survey (NHANES III) has collected descriptive data, physical measures, and biomedical samples on approximately 9,000 children at ages two months through six years with oversampling for blacks and Mexican Americans. The extensive data from NHANES III will be linked to the child's birth certificate to use the prenatal and birth information. NCHS is obtaining the certificates from the States and linking the data. Data analysis will require application of appropriate software for analysis of the complex multistage sample design of NHANES III for both descriptive and multivariate findings.

Major Findings: None available yet.

Significance to Biomedical Research and the Program of the Institute: Our ability to determine the health outcome of children classified as high risk at birth compared to other children has been extremely limited. Children who have been followed are seldom representative of even small areas. Normative comparisons for hypothesis testing of poor health outcomes have been based on scales developed from small, potentially biased populations. Use of this nationally representative sample including growth and other measures is a unique opportunity to provide norms for both the clinical and research community.

Proposed Course of Project: After NCHS obtains and links birth certificates, we will perform both descriptive and multivariate predictive analysis for publication in peer-reviewed journals. Other research using the blood and urine samples has been proposed.

Publications: None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02513-02 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

High Status and Risk Factors Analysis of Premature High-Risk Deliveries

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: M.D. Overpeck Epidemiologist EB, DESPR, NICHD
Other: A.C. Trumble Chief, Computer Sciences Branch CSB, DESPR, NICHD

COOPERATING UNITS (if any)

March of Dimes (J.Petrini, K.Damus)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.05

PROFESSIONAL:

0.05

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Trends toward increasing survival of infants born at less than 750 grams (about 1-1/2 pounds) have suggested higher prevalence of complications related to birth and continuing health deficits. Dr. Overpeck documented the status for the births, subsequent deaths, and survival in the U.S. birth cohort of 1983. She has provided background and technological support for staff of the March of Dimes (Joann Petrini) to complete the collaborative analysis to assess changes in survival and mortality of these extremely low birth weight infants. The analysis will compare the baseline years before introduction of surfactant therapy (1983), years incorporating the experimental phase of surfactant therapy (1987-88), and the latest years of available data which include births associated with both surfactant and antenatal corticosteroid therapy (1990-92). Deaths to infants born in the baseline years at weights less than 750 grams (about 24 weeks gestation) contributed about 25 percent of total infant mortality in 1983. Over 40 percent of deaths prior to six days of life were from these smallest least mature infants.



Project Description

Objectives: To assess changes in survival and mortality of live-born infants weighing less than 750 grams in the U.S.

Methods Employed: Summary data prepared by NCHS for release with the public use tapes of the 1983 Birth Cohort Linked Birth/Infant Data Set were analyzed for U.S. resident deaths of infants born in 1983. The latest data available from this source (probably 1987-89) is being compared for the total U.S. and local areas. Multivariate risk factor analysis will be completed.

Major Findings: Deaths in infants born at less than 500 grams (about 20 weeks gestation) contributed about 10 percent of total infant mortality in 1983. Deaths to infants born at less than 750 grams contributed about 25 percent. About 33 percent of all Black deaths occur to infants born at less than 750 grams; 22 percent of White deaths occur in this birth weight range. Over 40 percent of deaths prior to six days of life are from these smallest, least mature infants.

Significance to Biomedical Research and the Program of the Institute: The analysis of birth weight-specific mortality rates focuses attention on the timing of interventions needed to reduce infant mortality and to prevent such high risk deliveries. Birth weight-specific comparisons at the national and local levels will be made to assess differences in risk factors and outcomes. The time periods analyzed compare years encompassing the phases of testing and use of both surfactant and antenatal corticosteroid therapy.

Proposed Course: Changes in survival rates since 1983 will be analyzed with multivariate risk factor assessment. Publication in peer-reviewed journals and dissemination of local data through the March of Dimes is expected.

Publications: None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02514-02 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Childhood Drowning Deaths - A National Analysis of Death Certificate Information

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI: R.A. Brenner
Other: M.D. Overpeck

Staff Fellow
Epidemiologist

EB, DESPR, NICHD
EB, DESPR, NICHD

COOPERATING UNITS (if any)

Johns Hopkins University (G.Smith); Consumer Product Safety Commission
(A.McDonald)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Working in collaboration with the Consumer Product Safety Commission (CPSC) information is being abstracted from death certificates for all non-boat related drowning deaths in children and adolescents less than 20 years of age. The free text portion of the death certificate often includes important information that is not included in national mortality files. Of particular importance is the specific site of the occurrence (e.g. swimming pool, lake, irrigation ditch, etc.) as interventions to prevent drowning deaths are site specific. Results from this study will provide the first in depth look at the circumstances surrounding drowning deaths at a national level.



Project Description:

Objectives: To describe the epidemiology of drowning in the U.S. through the abstraction and analysis of death certificate information.

Methods Employed: Through an agreement with the Consumer Product Safety Commission, death certificates will be purchased from the fifty states and two major health jurisdictions (New York City and Washington, D.C.) for deaths in which the underlying cause of death is a nonboat-related drowning (E-code 910.0-910.9, 954, 964 and 984) and the age of the victim at the time of death is less than 20 years. Information from the purchased death certificates will be abstracted and coded to provide a uniform dataset for analyses of drowning deaths.

Data will be used in descriptive analyses of the circumstances of drowning, with particular focus on stratified analyses of the specific site of drowning by age at death and state of occurrence. Other variables of interest include sex, race, products associated with the death, and other details surrounding the event (e.g. the involvement of alcohol) abstracted from the narrative portion of the death certificate .

Major Findings: Results not yet available, however, a recent review of the literature and the epidemiology of drowning in the adolescent age group has been completed by two of the investigators on this study. The authors again identified the lack of data at the national level on the primary circumstances surrounding drowning deaths with over 70% of drownings in the adolescent age group being coded to the "other" or "unspecified" categories in national datasets.

Significance to Biomedical Research and the Program of the Institute: In the United States, drowning is the second most common cause of unintentional injury death among children and adolescents. In the one to two year old age group it is the single leading cause of injury death. Other groups at increased risk of drowning include adolescent males (particularly black adolescent males) and, in recent years, infants less than one year of age.

Interventions to prevent drowning deaths are dependent not only on the age of the victim but also on the specific location and circumstances surrounding the event. Unfortunately, national mortality data often do not provide sufficient information regarding the circumstances of these drowning deaths to allow injury prevention experts to design appropriately targeted interventions. In particular the specific site of the drowning (i.e. swimming pool, lake, bucket, irrigation ditch etc.) can not be identified from computerized mortality data because the E-Code (the ICD code for external injuries) is not specific for site of drowning. The hard copy of death certificates are a valuable source of information on the circumstances surrounding drowning deaths, including the specific site of the event.

This study will be the first to describe, at a national level, the specific circumstances surrounding drowning deaths thus enabling the development of appropriately targeted interventions.

Proposed Course of Project: As outlined in the interagency agreement with CPSC, death certificates for all drowning deaths received since January 1, 1994 have been retained by CPSC. CPSC has coded information from those drowning deaths that were not product related. (Coding of information from product related deaths is in the usual scope of work at CPSC.) Contracts with the 50 states and two major health jurisdictions (New York City and Washington, DC) for the purchase of death certificates have been modified to include all non-boat related drowning deaths as outlined in the interagency agreement. These modifications took effect in FY'95. As of June 1995, 998 death certificates were abstracted for deaths occurring in 1993 and 576 for 1994. Under the terms of the agreement, CPSC will continue coding this death certificate information through the end of FY'96. Due to delays in reporting (from the states to CPSC) it is anticipated that the most complete dataset will be compiled for the 1994 calendar year.

Publications:

Smith G, Brenner R. The changing risks of drowning for adolescents in the U.S. and effective control strategies. *Adoles Med: State-of-the-Art-Reviews* 1995;6:153-69.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02515-02 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Prevention of Childhood Injuries - Phase I Injury Surveillance

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	R.A. Brenner	Staff Fellow	EB, DESPR, NICHD
Other:	M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD

COOPERATING UNITS (if any)

Children's National Medical Center (P.Scheidt); D.C. Commission of Public Health; D.C. General Hospital; Georgetown University; Howard University; University of the District of Columbia

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☐ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

As one component of the "NIH/DC Initiative to Reduce Infant Mortality" a program of research addressing the threat of injuries and neglect to infants and toddlers in the District of Columbia has been developed. The study is divided into two phases. During phase one a comprehensive, city-wide injury surveillance system will be established. This surveillance will include all injuries in the target age group (birth through two years) that result in an emergency room visit, hospitalization or death. Information will also be obtained from the Department of Family Services on substantiated cases of abuse and neglect.

The information obtained in phase I will guide the design of specific targeted interventions, for the prevention of injuries, that will be implemented and evaluated in phase II. A randomized intervention trial of local community entities (neighborhoods or wards) is proposed in which epidemiologically based environmental interventions are applied to one group, behaviorally based interventions to prevent abuse and neglect to a second group and epidemiologic surveillance alone to a third group. As an essential component of injury prevention, injury surveillance system will continue for all three groups, throughout phase II.

The remainder of this report focuses on the phase I surveillance activities as detailed plans for the intervention phase are dependent on the results of the surveillance and thus have not yet been developed.

Project Description:

Objectives: 1) To collect citywide epidemiological data to define causes and patterns of injuries, both intentional and unintentional, that will enable investigators to develop effective intervention approaches in phase II of this study. 2) To determine the direct costs of intentional and unintentional injuries in the target population.

Methods Employed: Information will be abstracted from medical records and billing records of children ages 0-2 years who were seen in an emergency room or admitted to a hospital for an injury. All non-military hospitals in which children from the District of Columbia routinely seek care will be included in the surveillance. In addition, at three study sites a questionnaire will be administered to the parent or caretaker at the time of the ER visit or hospitalization. This questionnaire includes information on circumstances surrounding the injury event and other potential risk factors. Intake records from the Department of Family Services will be abstracted to provide information on cases of substantiated child abuse and neglect.

The leading causes of fatal and nonfatal injuries will be identified. Emergency room injury incidence rates, hospital admission rates and fatal injury incidence rates will be calculated. Injury rates will be compared between census tracts to identify communities in which children are at increased risk. Where community data exists on sociodemographic characteristics (e.g. maternal age) analyses will compare the characteristics of the injured population to those of the community to identify characteristics which place various subgroups at increased risk of injury.

Major Findings: Data not yet available for analysis.

Significance to Biomedical Research and the Program of the Institute: Injuries are the third leading cause of postneonatal death and the leading cause of death after the first year of life. Studies have shown that many of these injuries are preventable. Another serious problem is that of child neglect. In 1992, the D.C. Commission of Social Services investigated 4,886 families (with a total of 10,922 children and infants) for neglect. Approximately 25% of reported cases involve children less than three year of age.

The "NIH/DC Initiative to Reduce Infant Mortality" is aimed at addressing multiple causes and factors related to the high infant mortality rate in the District of Columbia. Among the types of research called for by the initiative is the development of interventions to prevent or reduce injuries. If interventions in phase II are effective, this program of research will not only reduce injuries among infants and young children of the District of Columbia but will also serve as a model program for other inner city communities.

Proposed Course of Project: Data collection for the Phase I study will begin in September of 1995 and continue through September of 1996. A detailed protocol of the Phase II intervention study will be developed based on the results of the initial six months of injury surveillance data. However, due to expected seasonal variations in the incidence and causes of injury, the protocol will not be finalized until a complete year of data are collected and analyzed.

Publications: None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02516-02 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

The Role of Bathtub Rings and Seats in Infant Drowning Deaths

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI: R.A. Brenner

Staff Fellow

EB, DESPR, NICHD

COOPERATING UNITS (if any)

Consumer Product Safety Commission (R.Rauchschwalbe); John Hopkins University (G.Smith)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Drowning is a leading cause of injury death among children in the United States. Recent studies have shown that, unlike other age groups, rates of death from drowning are actually increasing among infants. Approximately 40% of infant drownings occur in the bathtub. In this study we examine the involvement of bath rings and bath seats (products intended to support an infant in the sitting position during bathing) in drownings among infants and toddlers in the United States.

Using data from the Consumer Product Safety Commission, 21 drowning deaths, in which a bath seat or bath ring was in use at the time of submersion, were identified. Infants ranged in age from 5-15 months with a median age of 8 months. In a series of focus group discussions, parents reported that they were more likely to leave a child unattended momentarily in the bathtub if the infant was contained in a bath seat or ring. There was a reported lapse in adult supervision in 19 of 21 (90%) of the incidents. This study is the first in the medical literature alerting health professionals to the potential hazards of these products and reinforces the need to counsel parents on the dangers of leaving small children and infants unattended in the bathtub.



Project Description:

Objectives: To describe drowning and near drowning incidents involving the use of bathtub rings or seats.

Methods Employed: Data were obtained from the Consumer Product Safety Commission (CPSC) on deaths and near drownings involving bathtub seats/rings. Incidents were reported to CPSC through a number of sources including: both a voluntary and paid Medical Examiner's and Coroner's Alert Program; reports from a randomly selected number of hospitals that report all product-related emergency room injuries (NEISS); death certificates for product related incidents which are sent in from each of the fifty states and two major health jurisdictions (New York City and Washington, DC); a newspaper clipping service and a toll-free 800 line. Bathtub seat/ring incidents identified by CPSC Epidemiology staff are referred to the Division of Corrective Actions for in-depth investigation (IDI) assignment.

Reported incidents that occurred on or before December 31, 1994 and for which the IDI was completed by July 31, 1995 are included in this study. To be eligible for inclusion in this case series a bathtub seat had to be in use at the time of death or injury and the primary cause of death or injury had to be due to drowning or near drowning. Major findings are summarized below.

Major Findings:

1. Twenty-one deaths and six near drownings were identified. The first incidence occurred in August of 1983 and the most recent occurred in October of 1994.
2. The age at death ranged from 6 to 15 months, with a median age of 8 months.
3. There was generally a history of a lapse in adult supervision (either leaving the infant alone in the tub or in the care of an older child.) Among the fatal incidents, the lapse in adult supervision ranged from 0-35 minutes with a median of 5 minutes and a mean of 6.4 minutes.
4. Results from three separate focus panels showed that there is a false sense of security with bath rings and seats in that mothers/caregivers are more likely to feel comfortable leaving the infant alone in the tub for a few minutes when these products are in use.

Significance to Biomedical Research and the Program of the Institute: Drowning is an important preventable cause of death in childhood. Among infants and toddlers a large number of these drownings deaths occur in the bathtub. This study is the first in the medical literature to identify the involvement of bath rings and bath seats in drownings among infants and toddlers. It is hoped that, once alerted to the problem, health care professionals will warn parents and caregivers about the potential hazards of these products. Additionally it is thought that there is currently substantial underreporting of incidents involving these products. It is anticipated that after results from this study are published reporting of events (particularly non fatal events) involving these products will increase giving a more accurate estimate of the extent of the problem.

Proposed Course of Project: Preliminary findings were reported at the combined Society for Pediatric Research/American Pediatric Society/Ambulatory Pediatric Association meetings in May of 1995. A manuscript is currently being prepared and will be submitted for peer review.

Publications: None to date.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02517-02 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

First Week Changes in Birthweight and Other Neonatal Anthropometric Parameters

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	N. Tafari	Visiting Scientist	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief	EB, DESPR, NICHD
	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD
	J.F. Troendle	Staff Fellow	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

Department of Pediatrics, Addis Ababa University, Addis Ababa, Ethiopia

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.30

PROFESSIONAL:

0.30

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.)

Classification of newborns according to birthweight and gestational age is a useful clinical and epidemiologic tool to identify newborn infants at high risk for excess morbidity and mortality. In developing countries birthweight and gestational age cannot be measured directly. Moreover, preterm and low birth weight lose up to 10% of their birthweight during the first week. Using the statistical technique of recursive partitioning we recently demonstrated that head and chest circumference measurements accurately identified preterm, low birthweight newborns on data from Ethiopia. The rationale for employing other body measurements as surrogate for birthweight is dependent on their stability during the first week. We have also examined the results of serial measurements of head, chest, midarm circumference, body weight and length measurements in a cohort of 600 Ethiopian newborns in order to determine their pattern during the first week.

Project Description:

Objectives: To illustrate that measurements projected to serve as surrogates for birthweight such as head and chest circumferences are stable over the first week of life and have the same sensitivity and specificity in identifying preterm, low birthweight newborns.

Methods Employed: Body weight and length and, head, chest and midarm, were obtained on days 1, 3 and 7 on 300 infants with known gestational ages. Repeated measures of analysis of variance and recursive partitioning would be used to test the stability of surrogate measurements in predicting birthweight and gestational age.

Major Findings: Head, chest and midarm circumferences were stable while significant decline in birthweight was apparent.

Significance to Biomedical Research and the Program of the Institute: Effects of diverse health experiences of the mother prior to and during pregnancy are accurately reflected by birth weight and the duration of gestation. Identification of low birthweight and preterm babies would help in the understating of prenatal factors influencing the course of pregnancy and, fetal growth and development. Such classification tool may serve as an important adjunct in epidemiologic research in developing countries. It can also serve as a clinical tool for the identification and referral of high risk newborns for appropriate care. It may also assist clinicians caring for babies with unknown birthweight and gestational age.

Proposed Course of Project: Data analysis and manuscript preparation continue and are expected to be completed within the next fiscal year.

Publications: None this year.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02518-02 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Effectiveness of Rapid Plasma Reagin Screening in Gestational Syphilis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	N. Tafari	Visiting Scientist	EB, DESPR, NICHD
Other:	J.D. Clemens	Chief	EB, DESPR, NICHD
	M.R. Rao	Visiting Scientist	EB, DESPR, NICHD

COOPERATING UNITS (if any)

National Health Research Institute, Addis Ababa, Ethiopia (D. Zewdie)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.60

PROFESSIONAL:

0.60

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Congenital syphilis (CS) remains a major source of perinatal and infant mortality in sub-Saharan Africa and in high risk groups in the U.S. A major reason for the continued occurrence of CS is the inability of primary health care (PHC) services to provide a sustainable screening and treatment program. Dependence on centralized system of screening has been identified as the critical obstacle in the performance of the screening program. Moreover, a number of reports from the U.S. have drawn attention to the fact that current recommendations of prenatal treatment of gestational syphilis (GS) for prevention of CS may be inadequate.

As part of the national effort to improve the effectiveness of maternal and child health programs, the Ministry of Health of Ethiopia introduced a program of rapid plasma reagin "teardrop" (RPR-TD) test for screening and treatment of GS in PHC units without direct access to a medical laboratory. The RPR-TD was chosen over the standard venereal disease (VDRL) test because of several operational advantages including antigen stability and, possibility to carry out the test without the use of laboratory equipment such as the water bath, microscope or mechanical rotator. The test can be done and treatment can be given at initial contact thereby increasing patient and health care provider compliance. While a tendency towards reduction in fetal and infant mortality attributable to treatment could be demonstrated, there remained a large mortality gradient between the exposed and unexposed that appeared to be related in part to treatment failure, to diagnostic inaccuracy and to the presence of HIV co-infection.

Project Description:

Objectives: The aim of the study was to assess operationally the diagnostic performance of the rapid plasma reagin "teardrop" test (RPR-TD) in the prevention of congenital syphilis.

Methods Employed: The study was undertaken to evaluate a recently introduced program of the Ministry of Health of Ethiopia for screening and treatment of gestational syphilis (GS). The RPR-TD testing and treatment according to WHO recommendations of 2.4 million units of benzathine penicillin (BPG) was introduced in eight primary health care facilities offering prenatal care. In addition to routine clinical data, information on socioeconomic and demographic characteristics were obtained on all women undergoing the screening test. We assembled a cohort of 580 women who gave positive test on RPR-TD and who received BPG and 580 randomly selected women who were RPR-TD negative at the time of screening. The primary outcome measure was infant survival up to the age 12 weeks. We retrospectively evaluated the diagnostic performance of RPR-TD by comparing the results with VDRL microflocculation slide test and fluorescent treponemal antibody absorption test (FTA-ABS). All sera tested with VDRL and FTA-ABS were also screened for HIV with enzyme linked immunosorbent assays (ELISAs) and confirmed by western blotting.

Major Findings: Of the 3484 women screened with RPR-TD, 580 (17%) were positive. Comparison of the diagnostic performance of screening test with VDRL and FTA-ABS test results showed a sensitivity of 66% and specificity of 89%. The prevalence of GS according to VDRL/FTA-ABS test was 10% (357/3484). We followed 479 (83%) of the RPR-TD positive 513 (88%) of the RPR-TD negative pregnancies. Fetal and infant mortality was highest in those with GS/HIV co-infection (20/35 or 57%), and lowest in the group with no serological evidence for neither infection (17/617 or 5%). HIV infection was associated with 26% mortality and syphilis infection with 10% mortality. We postulate three hypotheses to account for failure to demonstrate significant reduction in fetal and infant mortality: failure to detect infection because of low sensitivity of the screening test, reduced efficacy of prenatal BPG therapy and, effect modification by HIV-1 co-infection.

Significance to Biomedical Research and the Program of the Institute: Syphilis and HIV are sources of excess perinatal and early infant mortality both in US women with high risk behavior and in African women living in rural poverty. The study offers a unique opportunity to measure the separate effects of failure of screening, treatment failure and, the effect modification of HIV co-infection on fetal and early infant mortality. The results of the study can indicate directions of future efforts towards improvement of the efficacy and effectiveness of screening and treatment of GS for the prevention of CS.

Proposed Course of Project: Analysis of the interaction between HIV and syphilis upon fetal and infant mortality will be undertaken in the coming year.

Publications: A manuscript has been submitted to the American Journal of Public Health.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER 201 HD 02519-01 EB																
PERIOD COVERED <p style="text-align: center;">October 1, 1994 through September 30, 1995</p>																		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Determination of Protection Level of Maternal Antibody to Group B Streptococcus																		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 45%;">F.-Y. Lin</td> <td style="width: 30%;">Research Medical Officer</td> <td style="width: 10%;">EB, DESPR, NICHD</td> </tr> <tr> <td>Others:</td> <td>J.D. Clemens</td> <td>Branch Chief</td> <td>EB, DESPR, NICHD</td> </tr> <tr> <td></td> <td>R.A. Brenner</td> <td>Senior Staff Fellow</td> <td>EB, DESPR, NICHD</td> </tr> <tr> <td></td> <td>Y.R. Johnson</td> <td>IRTA Fellow</td> <td>EB, DESPR, NICHD</td> </tr> </table>			PI:	F.-Y. Lin	Research Medical Officer	EB, DESPR, NICHD	Others:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD		R.A. Brenner	Senior Staff Fellow	EB, DESPR, NICHD		Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD
PI:	F.-Y. Lin	Research Medical Officer	EB, DESPR, NICHD															
Others:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD															
	R.A. Brenner	Senior Staff Fellow	EB, DESPR, NICHD															
	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD															
COOPERATING UNITS (if any) University of Alabama (B.Gray); Baylor College of Medicine (L.Weisman); Columbia University (J.Regan); University of Florida (P.Clark); UMDNJ (G.G.Rhoads); Magee Womens Hospital (B.Brozanski); Oakland Children's Hospital (P.Azimi)																		
LAB/BRANCH <p style="text-align: center;">Epidemiology Branch</p>																		
SECTION																		
INSTITUTE AND LOCATION <p style="text-align: center;">NICHD, NIH, Bethesda, Maryland 20892</p>																		
TOTAL STAFF YEARS: <p style="text-align: center;">1.2</p>	PROFESSIONAL: <p style="text-align: center;">1.2</p>	OTHER: <p style="text-align: center;">0</p>																
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input checked="" type="checkbox"/> (a) Human subjects</td> <td><input checked="" type="checkbox"/> (b) Human tissues</td> <td><input type="checkbox"/> (c) Neither</td> </tr> <tr> <td><input checked="" type="checkbox"/> (a1) Minors</td> <td></td> <td></td> </tr> <tr> <td><input checked="" type="checkbox"/> (a2) Interviews</td> <td></td> <td></td> </tr> </table>			<input checked="" type="checkbox"/> (a) Human subjects	<input checked="" type="checkbox"/> (b) Human tissues	<input type="checkbox"/> (c) Neither	<input checked="" type="checkbox"/> (a1) Minors			<input checked="" type="checkbox"/> (a2) Interviews									
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<input checked="" type="checkbox"/> (a2) Interviews																		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) <p>Group B Streptococcus (GBS) is the leading cause of neonatal sepsis and meningitis. Neonatal GBS diseases occur in two forms: early-onset and late-onset. Early-onset neonatal GBS disease occurs within 7 days of life, and the late-onset disease occurs after 7 days of life. Prevention of neonatal GBS through vaccination therefore could only be achieved through maternal transfer of antibodies to newborn infants. GBS capsular polysaccharide (Ia, Ib, II, III, IV, V) is a virulent factor and protective antigen. Vaccine based on capsular polysaccharide and protein conjugation is being developed at NICHD as well as other laboratories. Determination of a protective threshold would provide an important target for which candidate GBS vaccines administered during pregnancy should aim.</p> <p>The major objective of the study is to evaluate, for each GBS serotype, the pattern of the protective association between naturally acquired, serotype-homologous maternal serum IgG antibody titers and the risk of development of early-onset GBS disease.</p> <p>The surveillance began as of July 1995. The target is to accrue 140 early-onset GBS cases and 1120 controls (colonized infants) during the 30 month study.</p>																		

Project Description

Objectives:

- 1) The objective of this study is to evaluate the protective relationship between titers of maternal antibodies at delivery and the risk of Early-onset Group B Streptococcal (GBS) disease caused by GBS serotypes Ia, Ib, II, III, and V.
- 2) A secondary objective is to explore the relationship between GBS antibody titers in umbilical cord sera and the risk of early-onset disease, to assess the modifying role of placental transfer of antibodies.

Methods Employed: Two groups of infants will be recruited in this study: infants who have developed invasive GBS disease ("cases") and infants who have not developed disease despite being surface colonized ("controls"). This study will be conducted at 13 hospitals affiliated with 7 U.S. academic institutions.

Cases will be identified through active surveillance procedures established at each of the study sites. Contemporaneous controls will be assembled via surface cultures obtained from the throat, anus, umbilicus and ear canal on a systematic sample of infants born at these same sites.

Maternal and cord sera will be collected from these infants and their mothers. GBS isolates from case infants and from control mothers will be serotyped. Titers of IgG anti-CP antibodies to the infecting GBS serotype will be measured in maternal and cord sera, using a standardized assay. To characterize potentially confounding variables, patient charts will be reviewed. Cases and controls will be contrasted for the presence of antibody at various titers, both in simple analyses and in multivariable analyses that control for potentially confounding variables.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute: Establishment of a protective threshold of maternal antibody to serotype-specific polysaccharide of GBS will provide an important target for which candidate GBS vaccines (a vaccine that is being developed in the Institute) administered during pregnancy should aim.

Proposed Course of Project: The surveillance began in July 1995 and will continue through January of 1998.

Publications: None yet.

Contract Report: The study is conducted in collaboration with the University of Alabama in Birmingham, Baylor College of Medicine, Columbia University, University of Florida, Magee Womens Hospital of University of Pittsburgh, New Jersey School of Medicine and Dentistry and Children's Hospital Medical Center of Northern California under the following contracts: N01-HD-4-3214, N01-HD-4-3215, N01-HD-4-3216, N01-HD-4-3217, N01-HD-4-3218, N01-HD-4-3219, and N01-HD-4-3220. This project is supported by Westat that serves as a data coordinating

center under contract number N01-HD-5-3233. The 7 clinical centers are contracted to enroll GBS cases and colonized control infants. During the current fiscal year, a study protocol, a manual of procedure, and data forms were developed and the study enrollment began. During FY95 \$1,179,603 has been allotted to this project.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02520-01 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Maternal Caffeine Use in Pregnancy Outcome

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	M.A. Klebanoff	Research Medical Officer	EB, DESPR, NICHD
Others:	Richard Levine	Research Medical Officer	EB, DESPR, NICHD
	John Clemens	Chief, Epidemiology Branch	EB, DESPR, NICHD
	Rebecca DerSimonian	Statistician	BMSB, DESPR, NICHD

COOPERATING UNITS (if any)

Center for Human Toxicology, University of Utah (D.Rollins)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.15

PROFESSIONAL:

0.15

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

The role of maternal caffeine consumption in the pathogenesis of adverse pregnancy outcomes is controversial. Several studies have found that women who consume caffeine are at increased risk of spontaneous abortion and fetal growth retardation compared to non users. However, other, equally well-done studies have found no harmful effects of caffeine consumption. In addition, several studies have reported that caffeine is harmful only among women who smoke.

All previous studies of this question have relied on maternally-reported caffeine use; no studies have employed a biomarker for caffeine. This project will first validate the use of serum caffeine and its metabolites as a marker for caffeine intake, and will then study these serum markers as a risk factor for adverse pregnancy outcome.

Project DescriptionObjectives:

1. To determine whether serum levels of caffeine and/or its primary metabolites are correlated in a useful manner with caffeine intake.
2. To determine whether maternal caffeine use during pregnancy, as determined by serum levels of caffeine and/or its primary metabolites, is associated with spontaneous abortion.
3. To determine whether maternal caffeine use, as determined by serum levels of caffeine and/or its primary metabolites, is associated with reduced fetal growth.
4. To determine if increasing maternal caffeine consumption during pregnancy as determined by serum levels of caffeine and/or its primary metabolites, is associated with less favorable neuro-development in the offspring.
5. To determine if caffeine is more deleterious in smokers compared to non-smokers.

Methods Employed: In the first phase, the utility of serum caffeine and in paraxanthine to serve as a biomarker for caffeine intake will be evaluated. Women who completed a 24-hour dietary recall and had blood drawn at an unspecified time during working hours will be studied. The women were selected based upon their reported smoking and caffeine use. Thirty women were chosen at random from within each of 8 strata defined by 1) smoker/non-smoker and 2) caffeine consumption of <0.8 mg/kg a day; 0.8-1.49 mg/kg a day; 1.50-2.99 mg/kg a day; and ≥ 3.0 mg/kg a day.

Should the pilot data indicate that serum markers are useful, the main study will be done. In this study the first serum obtained from women experiencing a spontaneous abortion will be compared to serum obtained from women experiencing a live birth. The controls will be matched to the cases on age, time in pregnancy when the serum was drawn, study center, and smoking status. In addition, among controls, the first serum drawn after 26 weeks gestation will be evaluated to determine the association between caffeine and fetal growth retardation. Final models will include interaction times between caffeine and smoking. The exact marker for caffeine intake will depend on the pilot study results.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute: Caffeine is the most commonly used drug during pregnancy. Between coffee, tea, chocolate, and soft drinks, over half of all pregnant women are exposed to caffeine. If caffeine truly is harmful, then pregnant women should be encouraged to cut back or quit. If it is not harmful, then women may be reassured that they may safely continue its use. If caffeine is harmful only among smokers, then those women who do not smoke may be reassured, and those who smoke will have another reason to quit smoking.

Proposed Course of Project: The serum from the pilot study is currently being analyzed. The main study will proceed pending these results.

Publications: None yet

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02521-01 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Replacement of Pregnancy Losses: Interpregnancy Interval in Adolescence

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD
Others:	K.F. Yu	Statistician	BMSB, DESPR, NICHD
	P.M. Moyer	Computer Specialist	CSB, DESPR, NICHD

COOPERATING UNITS (if any)

NIDCD (HJ Hoffman)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.5

PROFESSIONAL:

0.5

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Adolescent age at first pregnancy is one of the single most important risk factors for adverse pregnancy outcomes. Adolescent women have higher rates of abortion, often this is utilized as a means of contraception. Adolescents also have a higher frequency of low birth weight and preterm births, as well as fetal and neonatal death(s). The important risk factors which have been shown to be associated with poor pregnancy outcomes in adolescents include biological and behavioral factors: inadequate prenatal care, maternal smoking, maternal drug and alcohol use, poor pregravid nutritional status, acute/chronic medical conditions, inadequate education, as well as shortened spacing (<two years) between successive pregnancies.

Shortened interpregnancy intervals increase the risk of adverse pregnancy outcomes. The association between birth spacing and pregnancy outcome may be related to biological factors such as, decreased uterine tonicity, depleted maternal nutritional reserves, and inadequate time to achieve physiological equilibrium prior to the next conception. One phenomenon which occurs in older women of reproductive age, and is associated with shortened interpregnancy intervals is reproductive compensation. This occurs as a result of perinatal loss in the previous pregnancy and the desire for women to replace that loss sooner.

The purpose of this study is to evaluate the relationship between prior perinatal loss (abortion, fetal and neonatal death) and the time between successive pregnancies in adolescent women. This objective will be achieved using a population-based analysis. An adolescent cohort was assembled using the Missouri linked data set of live births, late fetal and infant deaths from 1978 through 1990.

Project Description

Objectives: To evaluate the relationship between prior perinatal loss (abortion, fetal and neonatal deaths) and time between successive pregnancies in a cohort of adolescent women.

Methods Employed: An adolescent cohort of women was assembled from the Missouri linked data set of live births, late fetal and infant deaths from 1978 through 1990. Maternal and infant sibships were organized by a computerized linkage using maternal name, race, age, and parity. Higher-order births identified were linked to the previous birth by year/month of last birth/fetal death. Seven adolescent cohorts' ages <13-19 years were organized based on age at first pregnancy. Kaplan-Meier survival analysis was used for each age cohort as a function of interpregnancy interval. Age cohorts were compared with regard to time to subsequent pregnancy for up to three pregnancy events. Statistical significance of survival curve comparisons between each age cohort and a 24-29 year old reference group was based on the log rank chi square test. Cox regression analysis was employed to compare the hazards based on the outcome of the previous pregnancy controlling for race, maternal education, marital status, maternal smoking, pregravid body mass index (BMI), maternal medical risk factors, and prenatal care utilization. Interpregnancy interval was defined as the time from the previous birth to the next conception (defined by last menstrual period).

Major Findings: Preliminary results show:

- 1) The likelihood of a second pregnancy within one year of the first was greater for the youngest adolescents (up to 12%) compared to the reference group;
- 2) Women who began reproduction as adolescents were more likely to have higher parity (40-65% have at least three pregnancies) compared to the reference group;
- 3) The rates of abortion were greater for adolescents (30-50%) compared to the reference group (9-11%);
- 4) The risk of a subsequent pregnancy was 2.5 fold higher in adolescent women with a previous pregnancy loss compared to women with a previous term live birth;
- 5) Adolescent women tend to have shortened interpregnancy intervals (<1.5 years);
- 6) Among adolescent women, a prior pregnancy loss was associated with up to a twofold risk of a subsequent pregnancy within 1.5 years compared to women with a previous term live birth.

Significance to Biomedical Research and the Program of the Institute: This study will enhance our understanding of the phenomenon of reproductive compensation in adolescent women as a factor contributing to shortened spacing between successive births. This information will have a significant public health impact with regard to counseling adolescent women about proper birth spacing to decrease the risk of adverse pregnancy outcomes in this high risk population.

Proposed Course of Project: Completion of the final computer analysis and manuscript preparation will require an additional three to four months.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

201 HD 02522-01 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Intrapartum Antibiotic Prophylaxis against Early-Onset Grp.B Streptococcal Disease

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation)

PI:	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD
Others:	J.D. Clemens	Branch Chief	EB, DESPR, NICHD
	R.A. Brenner	Senior Staff Fellow	EB, DESPR, NICHD
	F.-Y. Lin	Research Medical Officer	EB, DESPR, NICHD

COOPERATING UNITS (if any)

University of Alabama (BM Gray, JB Philips); Baylor College of Medicine (LE Weisman); Columbia Univ.(JA Regan); Univ. of Florida (P Clark); Univ. of Pittsburgh (BS Brozanski, RL Sweet); Univ.Med.Dent.NJ (GG Rhoads); Med.Ctr.No.Calif.(PH Azimi)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.4

PROFESSIONAL:

0.4

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
- ☐ (a1) Minors
- ☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

Neonatal group B streptococcal (GBS) disease is the most common disease affecting newborn infants with an estimated incidence of 1.8 per 1000 live births. Twenty-five percent of neonatal GBS disease occurs in premature infants. GBS disease results in significant mortality and morbidity in newborns. Colonization of the infant occurs via vertical transmission from a colonized mother. Among pregnant women, the rate of GBS colonization is approximately 20-30%. Approximately 42-72% of infants born to colonized mothers will become colonized, and 1-2% of these infants will develop invasive GBS disease. A major factor which may modify the rate of colonization, and possibly the risk of invasive disease is intrapartum antibiotic prophylaxis given to women who are at definably high risk for early-onset GBS disease. This intervention strategy has been shown to be near absolute in efficacy trials in conferring protection; it seems probable that substantially lower levels of protection occur when recommended regimens are attempted in routine clinical practice. Supporting this notion is data indicating that intrapartum antibiotic prophylaxis fails to prevent up to 25-30% of cases of early-onset neonatal GBS disease. The purpose of this retrospective case-control study is to evaluate the effectiveness of intrapartum antibiotic prophylaxis given to high risk women in routine clinical obstetric practice with the intent to prevent early-onset neonatal GBS disease. This objective will be accomplished by the following: 1) assembled case and control infants will comprise all live births born to high risk women at the participating hospitals over three years from January 1, 1992 through December 31, 1994; 2) cases and controls will be assembled from a retrospective review of hospital laboratory logs; and 3) perform detailed chart abstraction of clinical and demographic data from the medical records of all eligible case-control maternal and infant pairs.

Project Description

Objectives: To assess the protection associated with the administration of antibiotics given during labor to high risk pregnant women as prophylaxis to prevent early-onset neonatal group B streptococcal disease (GBS).

Methods Employed: This is a retrospective case-control study designed to evaluate the effectiveness of intrapartum antibiotic prophylaxis given to high risk women in routine clinical obstetric practice with the intent to prevent early-onset neonatal GBS disease. Cases and controls will be assembled via retrospective review of hospital laboratory logs, hospital discharge diagnoses, and obstetric delivery logs. The sampling frame will comprise all live births occurring at the participating study sites between January 1, 1992 through December 31, 1994; infants born to high risk mothers will be potentially eligible for enrollment in the study. Case infants will be defined as those with documented early-onset GBS disease, and control infants will be defined as those with no documented early-onset GBS disease. Two Controls will be matched to each case based on the hospital of birth, date of birth within three months, and gestational age within two weeks. After assessment of eligibility for enrollment in the study, detailed chart abstraction of clinical and demographic data from the medical records of case and control maternal-infant pairs will take place. Chart abstraction forms designed for this study will be completed on all eligible case and control infants at each study site. These data will be sent to NICHD for data entry, data editing, and analysis.

Major Findings: None to date (project is under development).

Significance to Biomedical Research and the Program of the Institute: This study will greatly enhance our understanding of the effectiveness of intrapartum antibiotic prophylaxis intended to prevent early-onset GBS disease in newborn infants. Results from this study will allow better recommendations to be made regarding treatment of mothers and their newborns. In addition, this study of the effectiveness of intrapartum antibiotic prophylaxis will greatly assist in the proper interpretation of the protection conferred by maternal serum antibodies against GBS disease, an NIH sponsored study currently taking place at seven participating U.S. academic centers.

Proposed Course of Project: Three to four months will be required for data collection, and an additional two months for data entry, editing, and analysis.

Publications: None

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HD 02523-01 EB

PERIOD COVERED

October 1, 1994 through September 30, 1995

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)

Studies Based on Maternally Linked Birth Registries of Missouri and Utah

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

PI:	A.A. Herman	Visiting Scientist	EB, DESPR, NICHD
Others:	K.F. Yu	Mathematical Statistician	BMSB, DESPR, NICHD
	Y.R. Johnson	IRTA Fellow	EB, DESPR, NICHD

COOPERATING UNITS (if any)

NIDCD (H.Hoffman); Missouri Department of Health (G.Land, J.Stockbauer, W. Schramm); University of Utah (R.Ward, A.Fraser)

LAB/BRANCH

Epidemiology Branch

SECTION

INSTITUTE AND LOCATION

NICHD, NIH, Bethesda, Maryland 20892

TOTAL STAFF YEARS:

0.2

PROFESSIONAL:

0.2

OTHER:

0

CHECK APPROPRIATE BOX(ES)

- ☒ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
- ☒ (a1) Minors
- ☒ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

These studies focus on: 1) the impact of adolescent age at first pregnancy on later pregnancy outcome; 2) the relation between interpregnancy interval and later pregnancy outcomes; 3) the risk of repeating poor pregnancy outcomes. The data sets are unique in that all pregnancies of women from Missouri and Utah are linked into sibships. These data allow the analyst to examine longitudinal pregnancy data.



Project Description:

Objectives: 1) To examine the impact of adolescent age at first pregnancy and age greater than 35 years at first pregnancy on later pregnancy outcome; 2) the relation between interpregnancy interval and later pregnancy outcomes; 3) the risk of repeating poor pregnancy outcomes. The data sets are unique in that all pregnancies of women from Missouri and Utah are linked into sibships.

Methods Employed: Analytic methods include risk-based analysis of recurrent mortality and morbidity, life-table analyses and cox regression analysis, indirect standardization of mortality rates by birth weight and gestational age, examination of bivariate distributions using contour diagrams, fuzzy clustering of growth retarded and non growth retarded infants, non-parametric logistic regression, and other procedures applicable to population-based data. The epidemiologic approach is that of a "prospective" cohort design, which permits analyses by case-control, cross-sectional or longitudinal means.

Major Findings: Abstracts dealing with interpregnancy interval among adolescents (Society for Pediatric Research and Society for Pediatric Epidemiologic Research), increasing risk for perinatal mortality, preterm birth and intrauterine growth retardation among adolescents (Society for Pediatric Epidemiologic Research), sociodemographic profiles and adverse reproductive outcome (American Public Health Association), the recurrence of birth defects (American Society of Human Genetics), and growth retardation and SIDS have been presented in 1995. These results showed that: 1) adolescent women tend to have shorter interpregnancy intervals, 2) the shorter intervals have an adverse effect on pregnancy outcomes, 3) adolescents become pregnant more frequently than older women, 4) risks for perinatal mortality, preterm birth and growth retardation increases with parity among teenagers, 5) adolescents at increased risk for these outcomes have higher rates of poor pregnancy outcome than older women with comparable risk factors, 6) siblings of SIDS infants were not at increased risk for IUGR when maternal smoking was accounted for, 7) both similar and dissimilar birth defects recur with a statistically significant increased frequency, but context and paternity do not further increase the risk, and 8) paternal low SES had a greater influence on low birth weight than maternal low SES. The impact of low SES could not be explained solely by the frequency of high risk behavior among low income families.

Significance to Biomedical Research and the Program of the Institute: These studies will allow researchers to more fully explore the longitudinal impact of risk factors on pregnancy outcome. The repeater studies are among the first using United States data. Early interventions among high risk women may eventually lead to a reduction of preterm birth and IUGR.

Proposed Course of Project: The initial year of data analysis has been fruitful. Dr. Herman will continue to examine the longitudinal impact of risk factors, recurrence risk, the impact of low SES on pregnancy and the impact of early - or late first pregnancy on SES.

Publications: None yet.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 HD 02524-01 EB						
PERIOD COVERED October 1, 1994 through September 30, 1995								
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) Occupational Nonfatal Injuries in U.S. Children								
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and Institute affiliation) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: M.D. Overpeck</td> <td style="width: 33%;">Epidemiologist</td> <td style="width: 33%;">EB, DESPR, NICHD</td> </tr> <tr> <td>Other: K.A. Dunn</td> <td>IRTA Fellow</td> <td>EB, DESPR, NICHD</td> </tr> </table>			PI: M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD	Other: K.A. Dunn	IRTA Fellow	EB, DESPR, NICHD
PI: M.D. Overpeck	Epidemiologist	EB, DESPR, NICHD						
Other: K.A. Dunn	IRTA Fellow	EB, DESPR, NICHD						
COOPERATING UNITS (if any) U.S. Bureau of the Census (E.Lamas)								
LAB/BRANCH Epidemiology Branch								
SECTION								
INSTITUTE AND LOCATION NICHD, NIH, Bethesda, Maryland 20892								
TOTAL STAFF YEARS: 0.3	PROFESSIONAL: 0.3	OTHER: 0						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews								
SUMMARY OF WORK (Use standard unrounded type. Do not exceed the space provided.) <p>As many as 300 children a year may be killed while working in the U.S.; an unknown number suffer nonfatal injuries on the job. Current information on occupational injuries in children is based entirely on death certificates or workmen's compensation reporting. Both sources are known to contain extensive and biased underreporting for children. Many children are working illegally, "off-the-books", or in hazardous conditions including, but not limited to, those inappropriate for the child's health and developmental status. Determination of high risk occupations and incidence and severity of non-fatal injuries has been made a priority in the injury control and pediatric emergency treatment communities. Current policy questions on effects of 'school-to-work' programs require supporting baseline data on current work practices and injuries. NICHD staff are working collaboratively across Federal Agencies to determine the incidence of non-fatal injuries occurring to children in the workplace by occupational category exposure time. Dr. Dunn is completing a study of occupational exposure time for adolescents ages 15-18 from the Census Bureau Survey of Income and Program Productivity (SIPP). Her work will be used to provide unique and relatively unbiased exposure data for future epidemiological studies. Dr. Overpeck has prepared injury questions to be fielded in the SIPP in 1995 and later years. This will enable researchers to target interventions effectively by identifying both high risk children and high risk occupational categories.</p>								

Project Description: Research on adolescent occupational injury in the U.S. has been planned in three phases using the Survey of Income and Program Participation (SIPP). Exposure data for working adolescents is under development now by Dr. Kathleen Dunn. Questions on injuries to adolescents will be added to the SIPP for the current survey in the field and a new survey under development. The goal is to determine injury incidence and enable researchers to target interventions effectively by identifying both high risk children and high risk occupational categories.

Methods Employed: The (SIPP) is a continuous household survey fielded by the U.S. Census Bureau. A household is followed for 2 ½ years. Core questions and special topical supplements are administered at four month intervals. SIPP currently determines occupational status, category and time for all adults ≥15 years and tracks those who move. Limited injury questions have been included in the past. Dr. Overpeck has developed questions on injuries at ages 12-17 that will be included for a four-month period of 1995 with a Child Well-being Module sponsored by NICHD's Demographic and Behavioral Science Branch. She will also have occupational injury questions included for 50,000 households in the survey to begin in 1996. Injuries will be assessed for medical treatment and severity. Analysis through multivariate regression techniques appropriate for longitudinal complex multistage surveys will be performed.

Major Findings: None yet.

Significance to Biomedical Research and the Program of the Institute: As many as 300 children a year may be killed while working; an unknown number suffer nonfatal injuries on the job. Current information on occupational injuries in children is based entirely on death certificates or workmen's compensation reporting. Both sources are known to contain extensive and biased underreporting for children. Many children are working illegally, "off-the-books", or in hazardous conditions including, but not limited to, those inappropriate for the child's health and developmental status. Determination of high risk occupations and incidence and severity of non-fatal injuries has been made a priority in the injury control and pediatric emergency treatment communities. NICHD staff are working collaboratively across Federal Agencies to determine the incidence and severity of non-fatal injuries occurring to children in the workplace by occupational category exposure time. The ultimate goal is to enable researchers to target interventions effectively by identifying both high risk children and high risk occupational categories.

Proposed Course: Dr. Dunn's work will be used as part of her dissertation for a degree in Epidemiology at the University of North Carolina and will be submitted for publication. Dr. Overpeck will use the preliminary work on exposures prepared by Dr. Dunn to evaluate the injury data that will be available for the first time in late 1996.

Publications: None to date

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 HD 02525-01 EB
PERIOD COVERED October 1, 1994 through September 30, 1995		
TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.) World Health Organization Study of Health Behavior in School Children (WHO-HBSC)		
PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)		
PI: M.D. Overpeck Others: N.Krasnegor P.C. Scheidt	Epidemiologist Chief, HLBB IPA	EB, DESPR, NICHD HLBB, CRMC, NICHD HLBB, CRMC, NICHD
COOPERATING UNITS (if any) Substance Abuse and Mental Health Services Admin. (B.Rouse); Bar Ilan University, Israel (Y.Harel); WHO-HBSC Study Group (27 countries)		
LAB/BRANCH Epidemiology Branch		
SECTION		
INSTITUTE AND LOCATION NICHD, NIH, Bethesda, Maryland 20892		
TOTAL STAFF YEARS: 0.1	PROFESSIONAL: 0.1	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) Human subjects <input type="checkbox"/> (b) Human tissues <input checked="" type="checkbox"/> (c) Neither <input type="checkbox"/> (a1) Minors <input type="checkbox"/> (a2) Interviews		
SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.) The WHO coordinates a quadrennial survey that compares health behavior in adolescents in 27 countries. The survey is used to monitor and investigate patterns, determinants and effects of health related behaviors including smoking, substance use, and injury behavior. The U.S. has not participated yet. NICHD staff attended meetings with the international working group to learn about the process of comparing the data, results of the most recent international studies, and to apply to become a collaborating nation. The Substance Abuse and Mental Health Services Administration (SAMHSA) has already developed and funded a U.S. school-based survey to be fielded in Fall, 1995, that will meet the preliminary criteria for U.S. participation in the WHO-HBSC project. The successful completion and analysis of the U.S. survey and linkage to the existing data of the ongoing WHO surveys will allow the U.S. to compare behavior and related injury factors to data from 27 countries of Europe, Canada, and elsewhere. The Epidemiology and Human Learning and Behavior Branches of NICHD are collaborating with SAMHSA to conduct the data analysis for both the U.S. and international data sets. NICHD will focus particularly on injury comparisons. The data will prove invaluable for both international and domestic comparisons of injury and related behaviors in adolescents to target interventions since injury is the leading cause of death for children in the U.S.		

Project Description:

Objectives: The World Health Organization Study of Health Behavior in School Children (WHO-HBSC) is used to monitor and investigate patterns, determinants and effects of health related behaviors including smoking, substance use, and injury behavior. The U.S. has not been a participant. Collaboration with the Substance Abuse and Mental Health Services Administration (SAMHSA) will permit comparative analysis of both the U.S. and international data. NICHD will focus particularly on injury and related behaviors.

Methods Employed: The WHO coordinates a quadrennial survey that compares health behavior in adolescents in 27 countries. SAMHSA is collecting comparable data in a school-based study of U.S. adolescents. NICHD collaborators in the intramural and extramural programs will use descriptive and multivariate epidemiologic techniques appropriate for complex multistage complex surveys to prepare analytic papers.

Major Findings: None available yet.

Significance to Biomedical Research and the Program of the Institute: Injury is the leading cause of death for children in the U.S. NICHD has been active in promoting injury research as an emerging new endeavor through behavioral and epidemiological analyses and will emphasize this area in the collaborative analyses. The U.S. has learned much about risk factors and health outcomes through such international comparisons on infant mortality, aging, heart disease and health behaviors, for example. These data will prove invaluable for both international and domestic comparisons of injury and related behaviors in adolescents to target interventions appropriate to the U.S.

Proposed Course: NICHD has proposed joint funding by the intramural and extramural programs to support analysis of the SAMHSA survey data. The collaborative research team will complete the work through an interagency agreement with SAMHSA which has an on-going contract to perform the survey and complete the analysis.

Publications: None to date





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